LIPID STUDIES DURING PREGNANCY AND

IN INFANCY

by

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The work contained herein is the result of my own investigation, following initiation of a study into the hyperlipidaemia of pregnancy by Dr K. Barnes.

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ABSTRACT

This thesis is concerned with the hyperlipidaemias which occur during pregnancy and in infancy. It is presented in the form of 6 chapters, 5 of which contain a relevant introduction and section of methodology, preceded by a general introduction and review of relevant literature.

The hyperlipidaemia of pregnancy was manifested by mid-gestation. Using ultracentrifugation, the pattern of cholesterol and triglyceride distribution among the major lipoprotein fractions most closely conformed to the phenotype of primary (genetic) combined hyperlipoproteinaemia. Hypertension and pre-eclampsia exaggerate the triglyceride component, whilst pre-existing hypercholesterolaemia and renal disease also modify the pattern.

The hyperlipidaemia of pregnancy was further investigated using sterol balance techniques. The size of early plasma cholesterol increases was related to maternal weight gain. Bile acid excretion in the second trimester was related to plasma cholesterol and triglyceride concentrations, and may be increased compared to the first and third trimesters.

The next sections concern changes in the perinatal and infantile periods, with consideration of possible influences and their mode of action. Foeto-maternal stress was an important determinant of plasma triglyceride concentration in the infant at birth, whilst plasma cholesterol was related to maternal LDL-cholesterol concentration. Changes in plasma cholesterol and triglyceride were monitored during the first few days of life. The magnitude of the hyperlipidaemia was partly determined by the loss of weight during this early period.

A prospective study measured plasma lipid concentrations in 230 children at birth and at 12-22 months of age. The aim was to determine

the value of the measurement of umbilical cord plasma during infancy. The plasma lipid concentrations during infancy were influenced by the child's sex, weight and diet, and plasma cholesterol was related to his birth level. An hypercholesterolaemic child at birth was more likely to be hypercholesterolaemic during infancy, than one who was normocholesterolaemic at birth. However, the majority of hyperlipidaemic infants at 12-22 months were normocholesterolaemic at birth, but were more likely to have a family history of arteriosclerotic disease or hypercholesterolaemia.

Cholesterol metabolism was studied in the neonate and young infant using sterol balance techniques. Bile acid and neutral sterol excretion doubled during the first 3 weeks of life, becoming comparable to adult values (on a weight basis). Dietary cholesterol and the fatty acid composition profoundly affected the rates of excretion.

The final chapter is an applied problem concerning modification of the lipid composition of human milk. Milk linoleate content was doubled by substitution of polyunsaturated margarine and oils for butter and some of the saturated animal fat in the diet of mothers who were breast-feeding. The changes in milk fatty acid were correlated with maternal plasma changes, and the effect upon the infants' plasma cholesterol concentrations was monitored. The results are discussed in relation to the secretion of milk as well as changes in infant cholesterol metabolism.

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CHAPTER 1

INTRODUCTION

A. CHANGES IN PLASMA LIPIDS IN PREGNANCY AND THE PUERPERIUM

Historically the hyperlipidaemia of pregnancy has been known for many years, having been described by John Hunter in the eighteenth century, and by Rodier and Becquerel (1845) and Virchow (1847) (Boyd, 1934). Numerous studies have been carried out during the last 50 years, with increasing sophistication of techniques and better definition of the lipid fractions being investigated.

1. Normal Human Pregnancy

Cholesterol. The plasma cholesterol level has been reported to increase progressively during pregnancy and then either decrease before delivery (Oliver and Boyd, 1955; Watson, 1957; Hashmi and Afroze, 1972), remain elevated during the last trimester (de Alvarez *et al*, 1959; Pantelakis *et al*, 1964a,b) or continue to increase right up to term (Peters *et al*, 1951; Moses *et al*, 1952; von Studnitz, 1955; Vernet and Smith, 1961; Svanborg and Vikrot, 1965a). It has been suggested that the plasma cholesterol level may actually decrease during the first trimester of pregnancy (e.g., de Alvarez *et al*, 1959; Green, 1966). The level remains within the limits for the normal non-pregnant population, and if studies are not commenced early, in anticipation of pregnancy, such a decrease would be missed.

The change in plasma cholesterol in the post partum period has been variously described. Most authors have reported a slow, steady decrease in plasma cholesterol concentration following delivery (Watson, 1957; Konttinen *et al*, 1964; Svanborg and Vikrot, 1965b), but blood lipids have been reported as above normal limits several months post partum (Peters *et al*, 1951; Oliver and Boyd, 1955; Watson, 1957; de Alvarez *et al*, 1959). In one report, however, there was an initial decrease in plasma cholesterol, and then a rebound by the end of the first week (Oliver and Boyd, 1955).

Triglycerides. Most authors report a 2-3 fold increase by the third trimester or at delivery compared to the non-pregnant or early pregnancy values (Cramér *et al*, 1964/5; Konttinen *et al*, 1964; Renkonen, 1966; Svanborg and Vikrot, 1965a; Damiani *et al*, 1972; Knopp *et al*, 1973). The mode of increase is described as being steady throughout pregnancy (Peters *et al*, 1951; Knopp *et al*, 1973) with Svanborg and Vikrot (1965a) demonstrating a positive linear relationship between the log value of the plasma triglycerides and stage of gestation.

Plasma triglyceride concentration decreases rapidly following delivery. By day 5 post partum the concentration had halved (Svanborg and Vikrot, 1965b; Konttinen *et al*, 1964).

Phospholipids. Many of the studies mentioned above have included measurements of the plasma phospholipids. Total phospholipids increase during pregnancy, but Svanborg and Vikrot (1965a) have found differences in composition, the level of lysolecithin showing a marked decrease. Several authors have found a continuous increase until term or delivery (Peters *et al*, 1951; von Studnitz, 1955; de Alvarez *et al*, 1959; Svanborg and Vikrot, 1965a) whilst a decrease prior to term has been described by Oliver and Boyd (1955), Pantelakis *et al* (1964b) and Hashmi and Afroze (1972). The latter authors found that the level close to term was lower than at any other time during pregnancy.

The decrease in total phospholipids in the puerperium parallels that of cholesterol showing a slow steady fall following delivery (Konttinen *et al*, 1964; Svanborg and Vikrot, 1965b) or little immediate change (Oliver and Boyd, 1955; de Alvarez *et al*, 1959). The lysolecithin component abruptly increased during the first 4 days post partum (Svanborg and Vikrot, 1965b).

Fatty Acids. Free fatty acid concentration is increased during pregnancy. Fairweather (1971) found the levels to be higher than normal as early as the ninth week of gestation. There are other reports of elevated free

fatty acid levels in the third trimester and at delivery (Bleicher *et al*, 1964). The level of free fatty acid has been reported to decrease almost immediately after delivery of the placenta (Fairweather, 1965), with a continuing decrease over the first week after delivery (Becker *et al*, 1971; Bleicher *et al*, 1964).

Lipoproteins. In the 1950's, electrophoresis showed the changes in total plasma lipids to be equated with changes in the ratio of β and α lipoproteins during pregnancy, there being an increasing density of the β band coupled with an apparent change in migratory properties (Watson, 1957). The β/α ratio was uniformly increased (Russ *et al*, 1954; Oliver and Boyd, 1955; von Studnitz, 1955). Watson (1957) found that the mean β/α lipoprotein ratio had fallen towards normal by 6-8 weeks post partum, though was still above normal, supporting the finding by Oliver and Boyd (1955) that the cholesterol concentration and the ratio of β to α was still raised 20 weeks post partum.

Pantelakis *et al* (1964a,b) correlated lipoprotein changes during pregnancy derived by electrophoresis and ultracentrifugation. The pre-β band equated with lipoproteins of S_f 15-100 (having a density greater than 1.020). It was not increased in a normal pregnancy until after the sixteenth week, but in a diabetic pregnancy often earlier than this. The low density fractions, S_f 10-15 and S_f 3-9, increased later in gestation. Lipoprotein separation and measurement of the cholesterol and triglyceride components has recently been carried out by Knopp *et al* (1973) in conjunction with electrophoresis. They found all 3 major fractions (very low density lipoprotein, VLDL, low density lipoprotein, LDL, and high density lipoprotein, HDL) to be increased in the third trimester of pregnancy. Triglyceride was significantly increased in all fractions, whilst cholesterol increased only in VLDL and LDL.

Post Heparin Lipolytic Activity (PHLA). Since the triglyceride increase in pregnancy is of such magnitude, mechanisms which may affect triglyceride

metabolism have been investigated. PHLA has been considered to be an expression of the rate or efficiency of removal of triglyceride from the circulation. Meng and McGanity (1958) and Fabian *et al* (1958) found PHLA to be decreased in pregnancy, recovering during the puerperium. However, Knopp and Arky (1972) comparing pregnant normal and diabetic subjects were unable to demonstrate diminished PHLA in the third trimester of normal pregnancy, when a correction for its dilution in the larger plasma volume of pregnancy was made. The PHLA in diabetics was however significantly lower (diabetes, 3.96 ± 0.63; normal, 7.13 ± 1.05 m Eq ml FFA/µg heparin/hour), and coincided with differences in plasma triglyceride levels (271 and 169 mg/100 ml respectively).

2. Possible Effects of Diet, Race and Disease Studies conducted amongst pregnant women have generally shown that the hyperlipaemia of pregnancy is not affected by diet. Hansen *et al* (1964), in a group of 30 women, showed that the "degree of lipaemia in the last trimester of pregnancy" was independent of the maternal intake of calories, protein, carbohydrate, total fat, and saturated and unsaturated fatty acids. Vegetarians and non-vegetarians amongst a population studied in New Delhi showed similar increases in plasma lipids in the third trimester, but socio-economic differences were noted (Mullick *et al*, 1964). Similarly, use of a polyunsaturated diet did not influence the changes during pregnancy in either normo- or hyper-cholesterolaemic women (Green, 1966) and Moses *et al* (1952) were unable to influence plasma lipids by daily supplementation of the diet with 2 grams of cholesterol.

The socio-economic difference noted above by Mullick *et al* (1964) amongst the women in New Delhi bears further consideration. Damiani *et al* (1972) in Uganda compared African women with Asian and European women, the latter having a much higher social status than the former. Among non-pregnant controls the European and Asian women had significantly higher plasma cholesterol and phospholipid levels than the African women.

Whilst the Asian-European women showed an increase in plasma cholesterol during pregnancy, from 196 ± 35 mg/100 ml (mean ± S.D.) to 244 ± 42 in late pregnancy, the African women did not change (166 ± 27 and 157 ± 47 mg/100 ml respectively). Both groups showed an increase in plasma triglyceride (109 ± 59 to 241 ± 64 mg/100 ml, i.e. 221% amongst the Asian-European women, and 87 ± 8 to 177 ± 54 mg/100 ml, 204%, amongst the Asian women). A group of women from a low-income group in Nigeria had a plasma cholesterol level during pregnancy of 174 ± 6.9 mg/100 ml (mean ± S.E.M.) maximal at 24 weeks gestation, compared with 162 ± 2.6 mg/100 ml in the non-pregnant women. The plasma triglyceride levels in the same group were 97 ± 11.1 mg/100 ml at 24 weeks and 120 ± 5.9 at 36 weeks, compared with 47 ± 6.5 mg/100 ml in the controls. No data was available from either of these two studies regarding weight gain, skin-fold thickness, or nutritional status, although Taylor (1972) suggests that one of the major reasons for the absence of a rise in plasma cholesterol levels is inadequate caloric intake.

A further cultural group which shows little rise in plasma cholesterol during pregnancy is the Massai people. Mann and Shaffer (1966) studied 17 women at various stages of pregnancy, and found that there were no differences between their plasma cholesterol levels and that of age-matched non-pregnant women and the levels were all significantly lower than among American women. No data are available regarding diet, (though it was assumed to be similar to the remainder of the Massai, rich in animal fat and protein), apart from the statement that the women "were sleek and plump and showed every evidence of good nutrition". The skin-folds and weights "allowing for pregnancy gain" were similar to those in the non-pregnant women. Hormonal differences between these populations and others developing hypercholesterolaemia have been proposed as a cause (Taylor, 1972).

The occurrence of severe anaemia (a haemoglobin concentration less than 8 gm/100 ml) in pregnancy results in low plasma cholesterol levels throughout pregnancy when compared with non-anaemic pregnant women; but the level does increase during gestation, being maximal in the second trimester (Hashmi and Afroze, 1972). The numbers in this study were small, and no cause for the anaemias was documented.

Whilst there has been considerable interest in changes in metabolism which may accompany pre-eclampsia and eclampsia in pregnancy, there has been little agreement among reports concerning plasma lipids. The presence of higher plasma cholesterol, phospholipid and triglyceride levels has been reported (Boyd, 1934a,b; de Alvarez and Bratvold, 1961; Konttinen *et al*, 1964; Nelson *et al*, 1966), though not always reaching statistical significance. The triglyceride content of placentae from toxaemic and normal pregnancies may be different (136 mg/100 g and 89 mg/100 g respectively) (Nelson *et al*, 1966). Diabetics, not unexpectedly may show a marked increase in plasma lipids in pregnancy. Recently, total plasma triglyceride and cholesterol concentrations were shown to be higher in the third trimester in a group of diabetic women (plasma triglyceride - diabetic, 258 ± 11.4 mg/100 ml, n=21; normal, 186 ± 20.7, n=14; mean ± S.E.M.; $p < 0.02$). In a group of gestational diabetics the triglyceride level was higher than in normal pregnancies, but not statistically so, being 228 ± 16.1 mg/100 ml (Knopp *et al*, 1973). The plasma cholesterol was apparently lower in the latter group (193 ± 8.7 mg/100 ml) than in the normals (228 ± 9.0, $p < 0.02$), but higher in the diabetic group (245 ± 8.0, n.s.). Previous studies had shown no difference in plasma cholesterol levels between normal and diabetic pregnancies in the third trimester or at term (Vernet and Smith, 1961; Pantelakis *et al*, 1964a,b). Lipoprotein studies during diabetic pregnancy have indicated an increased pre-β concentration, both on electrophoresis (Vernet and Smith, 1961; Pantelakis *et al*, 1964a) and ultracentrifugation

(Pantelakis *et al*, 1964b). Furthermore, it is the more complicated diabetic pregnancies that show gross elevation of the pre-β band, which may remain raised for some time post partum (Vernet and Smith, 1961).

3. Animal Studies The effect of pregnancy upon plasma lipid concentrations has been studied in several animal species. Some of the results are shown in Table 1.1. There is a resultant hypocholesterolaemia in the subhuman primates (the baboon and the rhesus monkey) and in the rabbit, whilst a hypercholesterolaemia occurs in the dog and the rat. Plasma triglyceride concentration fluctuated widely in the studies in the rhesus monkey, but did increase towards term. Zilversmit *et al* (1972a) were unable to demonstrate a change in triglyceride levels in the rabbit.

In the puerperium, in the baboon plasma cholesterol levels increase following delivery and are elevated during lactation (169 mg/100 ml compared with 138 in the non-pregnant animal). In the rhesus monkey, the plasma cholesterol and triglyceride levels return to normal within 2 weeks of delivery, whilst the rats' plasma lipids return to control values on the third day post partum. The rabbit undergoes a rapid increase in plasma cholesterol level immediately after parturition, with a slight secondary decrease during lactation.

Triton WR 1339, a detergent which blocks the clearance of plasma triglyceride, has been used to study the mechanisms responsible for the changes in plasma lipids during pregnancy. Zilversmit *et al* (1972a) found the rate of secretion of cholesterol into rabbits' plasma to be 10.1 ± 1 mg/100 ml/hour in pregnant animals compared with 7.9 ± 0.6 mg/100 ml/hour in non-pregnant rabbits. Otway and Robinson (1968), in the rat, showed a progressive increase in entry of triglyceride-fatty acid into plasma as body weight increased with pregnancy coupled with a low adipose tissue clearing-factor lipase activity.

TABLE 1.1

Plasma Lipid Changes During Pregnancy and

	Animal	Length of Gestation	During Pregnancy	
			Plasma (mg/100 ml)	Cholesterol
Tietz *et al* (1967)	Dog (Beagle)	63 days	175 (control)	↑ 75%
Knobil *et al* (1957)	Rat	22 days		↑ 150%
Otway and Robinson (1968)	Rat			
Martin *et al* (1971)	Rhesus Monkey	168 days	145 (control)	↓ 50%
van Zyl (1957)	Baboon	196 days	138 (control)	↓ 70%
Zilversmit *et al* (1972)	Rabbit	32 days	≃75 (control)	↓

in the Puerperium: Animal Studies

During Pregnancy		In the Puerperium			
Plasma (mEq/100 ml)	Triglyceride	Plasma	Cholesterol	Plasma	Trigylceride
0.29	↑ 4-fold			↓ At parturition	
118	↑ Slightly	↓ Normal after 1 week		↓ Normal within 2 weeks	
		↑ And remains elevated during lactation			
No change		↑ After parturition then ↓ slightly during lactation			

B. THE EFFECT OF SEX STEROIDS UPON PLASMA LIPIDS

1. Plasma Lipids

Sex Differences. The effect of gestational hormones upon plasma lipids has been known for some time, the hyperlipidaemia of pregnancy having been recorded in the mid-eighteenth century (Boyd, 1934), with more recent observations on the effect of endogenous or administered hormones on lipid metabolism. The apparent sex difference in the incidence of atherosclerotic disease promoted interest in plasma cholesterol levels particularly. One further development has fostered recent interest in the possible mechanism of these hyperlipidaemias, namely the increased incidence of thrombotic disease among young women using oral contraceptive preparations (Vessey and Doll, 1969; Oliver, 1970; Hurtig, 1973).

A difference in plasma cholesterol levels between males and females is demonstrable from birth, the concentration being significantly higher in the umbilical cord samples of female infants (Barnes *et al*, 1972; Darmady *et al*, 1972), although smaller studies have failed to show a statistical difference. No difference was found between school age girls and boys in the Evans County study (Hames and Greenberg, 1961), whilst in an Australian population, school boys showed an adolescent increase in plasma cholesterol concentration earlier than the girls (Godfrey *et al*, 1972). Several studies have shown that the more rapid rise in plasma cholesterol concentration in the third decade of life is responsible for the higher level in adult males than adult females (e.g. Kannel *et al*, 1964; Prior and Evans, 1970). This difference is maintained for some 2 to 3 decades, but subsequently the level in the female increases, so that menopausal and post-menopausal values in women are equal to or greater than those in men of similar age.

As with plasma cholesterol, there is an increase in plasma triglyceride concentration with increasing age. Schaefer (1964) showed that triglyceride values in females are generally lower than in males of

the same age until the mid-sixties. Carlson and Lindstedt (1968) showed similar differences between men and pre-menopausal women.

The changes in plasma cholesterol and triglycerides seen with age are reflections of changes in lipoprotein concentrations. The pattern seen at birth is one of almost equal concentrations of HDL and LDL with very low levels of VLDL (Table 1.8). The relatively greater increase in plasma lipids in men during the third and fourth decades is due to increasing levels of LDL and VLDL (Fredrickson *et al*, 1967), a similar increase occurring in the female population after menopause. The concentration of HDL is higher in women throughout adult life.

The Effect of Castration. Since the change in plasma lipids that occurs with the menopause has been attributed to declining ovarian function, the effect of premature oophorectomy on plasma lipids has been studied. The effect is generally more pronounced than that observed during natural menopause.

The investigation of women 20 years after bilateral oophorectomy revealed a mean plasma cholesterol level of 251 mg/100 ml compared to 217 mg/100 ml in a comparable group who had undergone only unilateral oopherectomy (Oliver and Boyd, 1959). Similar results were found by Robinson *et al* (1957), although the age range of this population was rather wide (31-70 years). The age of the population is very important: among women who had undergone an hysterectomy only (the control group), there was a significant correlation between plasma cholesterol concentration and age (Aitken *et al*, 1971). No such relationship was present in the oophorectomized women of comparable ages. However, if the groups were subdivided into those younger than and older than 45 years of age, only the younger group of oophorectomized women had significantly higher plasma cholesterol levels than the controls (292 and 244 mg/100 ml respectively). The levels in the older group were 305 and 299 mg/100 ml respectively. Whilst there was no difference in the

levels of triglyceride, the slopes of the regressions of age on concentration in the 2 groups were significantly different, women with intact ovaries having a greater rise in plasma triglyceride with age than those who had been oophorectomized. Treatment of a subgroup of the oophorectomized women with oestrogen reduced the plasma cholesterol from 322 mg/100 ml to 268 after one year. This was accompanied by an increase in triglyceride levels from 97 mg/100 ml to 123 mg/100 ml. These changes are similar to those reported by Robinson and Le Beau (1965) for 40 post-menopausal women given low dose equine oestrogen for one month.

Castration in males also influences lipid metabolism. Early studies by McCullagh and Renshaw (1934) and Hamilton *et al* (1956) suggested that post-pubertal orchidectomy resulted in an elevation of plasma cholesterol levels. However, Furman *et al* (1957, 1958) could demonstrate no difference in total plasma cholesterol amongst middle-aged or elderly eunuchs, whilst the level was significantly lower in the group aged 21-30 years. More interesting, however, was the observation that in all age groups the levels of LDL were lower and HDL higher than in age-matched normal male controls.

The Effect of Exogenous Oestrogens. Oestrogens alone decrease the plasma cholesterol concentration in both men and women, in the latter both pre- and post-menopausally (Eilert, 1953; Russ *et al*, 1955; Robinson and Le Beau, 1965; Aitken *et al*, 1971). This applies also in hypercholesterolaemic men (Oliver and Boyd, 1956a,b). Plasma triglyceride levels, noted to be raised in post-menopausal women (Sznajderman and Oliver, 1963) were further increased by exogenous oestrogen (Robinson and Le Beau, 1965), and were increased in the group of oophorectomized women studied by Aitken *et al* (1971). If there is pre-existing elevation of plasma triglyceride, oestrogens will accentuate this (Zorilla *et al*, 1968).

Administration of oestrogen alone, both natural and synthetic, has resulted in all populations in a decrease in plasma LDL and a concurrent increase in HDL (Oliver and Boyd, 1954, 1956a,b; Hood, 1959; Robinson and Le Beau, 1965).

Combined Oestrogen-Androgen Preparations. These are most commonly taken by women as oral contraceptives. They have produced inconstant changes in plasma cholesterol levels, but have invariably increased the plasma triglyceride concentration (Table 1.2). The latter usually remains within the broad limits of normality (i.e. <150 mg/100 ml). Since administration of oestrogen alone will produce a similar change in the triglyceride concentration, the increase has been attributed to the oestrogenic component alone (Hazzard *et al*, 1969). The more "oestrogenic" the preparation, the greater the triglyceride change is likely to be (Stokes and Wynn, 1971).

The lipoprotein changes are also governed by the relative potencies of the individual components. The "oestrogenic" component increases VLDL (Wynn *et al*, 1966). Changes in HDL are variable; the more "androgenic" the activity, the lower the HDL concentration will become, and the higher the LDL and total plasma cholesterol level will be (Table 1.3).

The nature of the androgen is very important. Administration of naturally-occurring androgens (testosterone and Δ-4-androstenedione) in combination with oestrogen will not reverse the oestrogen shift (Hood, 1959). However, orally active androgens, such as methyltestosterone and 19-nortestosterone, result in an increase in LDL with a concurrent decrease in HDL (Oliver and Boyd, 1956a; Hood, 1959; Cramér, 1961, 1962).

Anabolic-Androgenic Steroids and Progestational Oral Contraceptives. With mounting evidence of alterations in lipid metabolism accompanying the administration of mixed oestrogen-progestin oral contraceptives, it has become desirable to find a pure progestational compound, hormonally active

TABLE 1.2

Plasma Lipid Changes in Women During Treatment With Oral Contraceptive Preparations

	Preparation	Control Period n	Control Period Cholesterol*	Control Period Triglyceride*	Treatment Period n	Treatment Period Cholesterol	Treatment Period Triglyceride	Duration
MIXED CONTRACEPTIVES								
Aurell *et al* (1966)	Anovlar		179	62		223	101	
Wynn *et al* (1966)	Various	38	181 ± 30	60	58	198 ± 34[1]	107	
Brody *et al* (1968)	Anovlar	9	238	65	8	205 ± 10[2]	73 ± 7	6 months
						240 ± 9	80 ± 8	12 months
	Ovulen	9	211	74		226 ± 13	121 ± 8	6 months
	Volidan	6	222			225 ± 15	110 ± 11	6 months
	Provest	10		45			64	
Hazzard *et al* (1969)	Various	54	181 ± 41	77 ± 31		194 ± 36[1]	116 ± 45	
Wynn *et al* (1969)	Orthonovin	51	184	134		197	171	6 months
Barton *et al* (1971)						206	180	12 months
	Norinyl-L	118	203	117		188	153	6 months
						200	156	12 months
Kekki and Nikkilä (1971a)	Various	17	207 ± 26	64 ± 22	13	193 ± 61[1]	98 ± 32	
Rössner *et al* (1971)	Anconcene	12	186 ± 6	81 ± 8		209 ± 10[2]	150 ± 10	
Stokes and Wynn (1971)	Various:							
	50 µg oestrogen	116	179 ± 35[1]	68 ± 23[1]	256	191 ± 39[1]	97 ± 35	
	100 µg oestrogen				204	197 ± 37[1]	113 ± 35	
Kuku and Akinyanju (1973)	Orthonovin Ovral	40	163 ± 32[1]	47 ± 12[1]	100	205 ± 29[1]	109 ± 26	
PROGESTIN ONLY								
Barton *et al* (1971)	Normenon	101	204	130		211	114	
Glueck *et al* (1972b)	Quinquestanol	21		68 ± 25			56 ± 30	

*Plasma concentration: mg/100 ml

[1]Mean ± S.D.

[2]Mean ± S.E.M.

TABLE 1.3

Plasma Lipoprotein Changes in Women During Treatment with Oral Contraceptive Preparations

	Preparation	Lipoprotein	Control Period		Treatment Period	
			Cholesterol*	Triglyceride*	Cholesterol	Triglyceride
Aurell *et al* (1966)	Anovlar	LDL	124	48	151	73
		HDL	60	12	46	18
Rössner *et al* (1971)	Anconcene	VLDL	8 ± 2	38 ± 6**	14 ± 2	65 ± 9
		LDL	100 ± 7	25 ± 3	106 ± 9	43 ± 4
		HDL	67 ± 5	10 ± 2	74 ± 8	34 ± 3
			Percentage distribution			
Kuku and Akinyanju (1973)	Orthonovin	VLDL	10.8 ± 1.2		17.8 ± 2.1	
	Ovral	LDL	64.3 ± 3.8		69.0 ± 4.6	
		HDL	24.1 ± 1.7		14.4 ± 1.3	

*Plasma concentration: mg/100 ml

**Mean ± S.E.M.

TABLE 1.4

Composition of Oral Contraceptive Preparations Used in Plasma Lipid Studies

Brand Name	Manufacturer	Oestrogen		Progestigin	
Anconcene	Merck	Mestranol	0.1 mg	Chlormadinone	3 mg
Anovlar	Schering	Ethinyl Oestradiol	0.05 mg	Norethisterone Acetate	4 mg
Norinyl-L	Syntex	Mestranol	0.05 mg	Norethisterone Acetate	1 mg
Normenon		—		Chlormadinone	0.5 mg
Orthonovin	Ethnor	Mestranol	0.1 mg	Norethisterone Acetate	2 mg
Ovral	Wyeth	Ethinyl Oestradiol	0.05 mg	Norgestrol	0.5 mg
Ovulen	Searle	Mestranol	0.1 mg	Ethynodiol Acetate	1 mg
Provest	Upjohn	Ethinyl Oestradiol	0.05 mg	Medroxyprogesterone	10 mg
"Quinquestanol"		—		Quinquestanol	0.3 mg
Volidan	Allen and Hanburys	Ethinyl Oestradiol	0.05 mg	Megestrol Acetate	4 mg

in terms of its contraceptive properties but not disturbing other metabolic processes. Progesterone and testosterone alone generally have little effect on plasma triglyceride or cholesterol levels in normal subjects (men or women) or in those with moderately raised plasma cholesterol levels (Oliver and Boyd, 1956; Svanborg and Vikrot, 1966). However, this is not the case with synthetic, orally active androgens. Glueck *et al* (1969, 1971a) using the anabolic-androgenic compound, Norethindrone acetate, lowered the plasma triglyceride concentrations of normal subjects as well as in patients with familial types III, IV and V hyperlipoproteinaemia. This followed the striking fall in plasma triglycerides in a young woman with familial type V hyperlipoproteinaemia, who was given the steroid for menstrual irregularity. By contrast, exogenous oestrogen may severely exacerbate symptoms in a familial type V hyperlipoproteinaemia patient (Glueck *et al*, 1970).

Other gestational oral contraceptives which have been developed include chlormadinone and quinquestanol acetate. They share the properties of norethisterone in that there may be a slight reduction in plasma triglyceride, but cholesterol remains unchanged (Table 1.2).

2. Possible Mechanisms of Action and Associated Hormonal Changes

Triglyceride Metabolism. There are marked differences in triglyceride kinetics between men and pre-menopausal women. Nikkilä and Kekki (1971), using normolipidaemic subjects, showed that whilst triglyceride turnover was the same in both sexes, women had a lower K_m than men on the basis of Michaelis-Menton kinetics. That is, at any given level of triglyceride influx women showed a more efficient removal system (a higher enzyme-substrate affinity), and therefore a lower plasma triglyceride. Reaven *et al* (1965) had previously shown that triglyceride turnover, as measured by the disappearance of labelled plasma triglyceride-fatty acid may obey the Michaelis-Menton equation. The sex difference in triglyceride kinetics also seems to apply in hypertriglyceridaemic subjects (Olefsky *et al*, 1974).

Whilst the removal of triglyceride has yet to be defined as a function of one enzyme system, the use of K_m in this context is as a descriptive, conceptual term, and is more properly defined as triglyceride concentration at half-maximal production or removal rates.

Use of combined oral contraceptives doubled plasma triglyceride production (Kekki and Nikkilä, 1971a; Kissebah *et al*, 1973), as did an oestrogen preparation alone (Kissebah *et al*, 1973). The increase in plasma triglyceride concentration was less than predicted from a simple increase in production (Kekki and Nikkilä, 1971a), and was sustained for up to 6 weeks after discontinuing steroids. Calculation of the removal rate of triglyceride from plasma in the subjects on mixed oral contraceptives showed the K_m to be greatly decreased (Kekki and Nikkilä, 1971a; Kissebah *et al*, 1973). Clearance of Intralipid, a commercial fat emulsion preparation was also increased (Kissebah *et al*, 1973). The turnover of plasma triglyceride was directly related to the length of oral contraceptive treatment in both studies. Whilst oestrogen alone increased plasma triglyceride turnover, it did not alter the clearance rates of endogenous or exogenous triglyceride (Kissebah *et al*, 1973). However, treatment with megestrol or progesterone produced the opposite effect viz: of increased clearance with no change in production rate. This would account for the observed changes in plasma triglyceride with the administration of oestrogen and androgen respectively. Oxandrolone, an anabolic androgen, also increases VLDL-triglyceride turnover in patients with familial type IV hyperlipoproteinaemia (Glueck *et al*, 1973a).

Post-Heparin Lipolytic Activity (PHLA). Several reports have been published describing marked reduction in PHLA in women on mixed oral contraceptives (Hazzard *et al*, 1969; Ham and Rose, 1969; Adams *et al*, 1970), or whilst taking oestrogen alone (Hazzard *et al*, 1969). The decrease has been of the order of 30-50% below the normal range and comparable with values recorded in familial type I hyperlipoproteinaemia

(Fredrickson *et al*, 1963), the lipidaemia of uncontrolled diabetes (Bagdade *et al*, 1967) or that occurring in myxoedaema (Porte *et al*, 1966).

PHLA was found to be normal in plasma samples taken 5 minutes after the injection of heparin, but depressed after 40 minutes in patients taking oral contraceptives (Rössner *et al*, 1971), Kissebah *et al* (1973) also found PHLA to be normal in women taking oral contraceptives, which is in keeping with the evidence now available that progesterones and the progestogenic component of oral contraceptives may improve clearance of circulating triglyceride. The variability in PHLA findings may be due to the conditions under which the studies were carried out and the differences in the oral contraceptives.

Other steroid hormones also affect PHLA. For instance, the lowering of plasma triglyceride in type V hyperlipoproteinaemic women with Norethindrone acetate was accompanied by an increase in PHLA into the normal range (Glueck *et al*, 1969, 1971a). However, a structurally related progestational oral contraceptive, quinquestanol acetate, given to normal subjects for one year, did not produce any change in PHLA (Glueck *et al*, 1973a). An earlier report showed a slight decrease in plasma triglyceride (Glueck *et al*, 1972b) (Table 1.2). Oxandrolone, another anabolic androgen, also increased PHLA in patients with familial type IV hyperlipoproteinaemia (Glueck *et al*, 1971b), with concurrent changes in triglyceride kinetics (Glueck *et al*, 1973b), but no correlation could be shown between triglyceride clearance rates and PHLA.

Insulin, Glucocorticoids and Growth Hormone. The interrelationship between the changes in carbohydrate and lipid metabolism during treatment with oral contraceptives has proved extremely complex. An increase in the fasting level of immunoreactive insulin in subjects on oral contraceptives has been reported (Spellacy *et al*, 1967; Yen and Vela, 1968; Hazzard *et al*, 1969). The increase of some 40% was attributed to the oestrogen component, as administration of oestrogen alone resulted

in a similar increment (Hazzard *et al*, 1969). However, other reports have found the level to be unchanged (Javier *et al*, 1968; Spellacy *et al*, 1968; Wynn and Doar, 1969). Similarly, changes in response to both oral and intravenous glucose tests have been at variance, with higher insulin levels being found by Spellacy *et al* (1967, 1968) and Yen and Vela (1968). Some of the differences may be due to the duration of treatment with the steroid preparations, as Javier *et al* (1968) noted that whilst insulin was high during oral glucose tolerance testing early in treatment, levels tended to become low after prolonged therapy. Similarly, Wynn and Doar (1969) were able to show higher insulin levels in response to oral and intravenous glucose in women who had recently commenced taking oral contraceptives, but no such difference was present in a second group who had been taking the preparations for a longer period of time (2-72 months).

Wynn and Doar (1966) have suggested that the impaired glucose tolerance associated with use of oral contraceptives is in fact a "steroid diabetes". Oestrogen therapy, in the dosage used in oral contraceptives, increases plasma cortisol levels three-fold (Burke, 1969). The increase is mainly within the protein-bound fraction, but this does have biological activity (Matsui and Plager, 1966).

Growth hormone is also reported to be increased during oral contraceptive therapy. Levels are raised during treatment both in fasting women (Spellacy *et al*, 1967a) and in response to hypoglycaemic stress such as produced by insulin (Spellacy *et al*, 1967b). This is consistent with sex differences in growth hormone levels both at rest and in response to provocation (e.g. Unger *et al*, 1965; Merimee *et al*, 1966).

C. PLASMA LIPIDS IN THE FOETUS, NEONATE AND YOUNG CHILD

1. Plasma Lipid Levels in the Young

Mammalian young are born with plasma cholesterol levels lower than those in the adult of the same species. A representative sample of these is shown in Table 1.5, summarizing some

TABLE 1.5

Changes in Plasma Cholesterol and Triglyceride Concentration in the Young Animal

	Species		Plasma Lipid Concentration (mg/100 ml)				
			Foetus	Newborn	Suckling	Weanling	Adult[1]
Carroll (1964)	Rat	Cholesterol			109 (99-127)	71 (59-80)	52 (28-76)
Friedman and Byers (1961)	Rabbit	Cholesterol	102 (82-173) (n=13)	121 (62-178) (n=33)	258 (127-478) (n=6)	51 (37-104) (n=14)	45 (10-80)
		Triglyceride	50 (34-118)	259 (42-584)	167 (54-494)	73 (23-194)	
Shope (1929)	Guinea-Pig	Cholesterol		65[2]	231[2]	63[2]	32 (21-43)
Carroll *et al* (1973)	Calf	Cholesterol		<25 (n=6)	≃100 (n=6)	≃50 (n=6)	110 (8-212)
	Lamb	Cholesterol		50 (n=6)	≃ 70 (n=6)	≃40 (n=6)	
	Pig	Cholesterol		50 (n=11)	135 (n=11)	70-100 (n=11)	(152-154)

[1] From "Blood and Other Body Fluids" (1961) ed. D.S. Dittmer

[2] Guinea-pigs: mean value of 4-8 animals

of the published values for various species. The changes which occur in the young with increasing age are also shown. The human is no exception to this rule, but the pattern of subsequent change with growth is at slight variance with other species. Some values published during the last 15 years have been chosen to illustrate this point (Tables 1.6 and 1.7).

Experimental Animals. All animals illustrated in Table 1.5 develop hypercholesterolaemia when they are suckling. The plasma levels increase rapidly after birth, so that most species show significant changes within the first week. The levels may continue to rise over successive weeks (e.g. Carroll *et al*, 1973) and/or remain at a plateau until weaning takes place. The association of weaning with the diminution in plasma cholesterol levels has led many to search for and implicate dietary components as responsible for the changes. For instance, Shope (1929), in reporting the hypercholesterolaemia in the suckling animal, considered that the cholesterol content of colostrum was responsible. Evidence for the role of dietary cholesterol comes from supplementation experiments, such as the hypercholesterolaemia in calves that was aggravated when cholesterol was added to milk (Wiggers *et al*, 1971). Friedman and Byers (1961) demonstrated the importance of both milk fat and cholesterol in raising the suckling rabbit's plasma cholesterol. The suckling rat reacts slightly differently, in that milk fat alone will produce hypercholesterolaemia (Harris *et al*, 1966). This suckling animal is also interesting in that its cholesterol does not fall with the addition of ethyl linoleate, though a fall occurs when residual bulk, in the form of cellulose, is added to the diet.

The effect of the combination of some or all of these factors may be seen in early or delayed weaning of pigs: those animals that have no access to solid food prior to weaning show an abrupt fall in the plasma cholesterol level on weaning, whereas piglets that during suckling have

early access to the mother's feed show a fall in plasma cholesterol levels before weaning (Carroll *et al*, 1973). The same authors compared the response of calves fed whole milk or skim milk. The plasma cholesterol of the latter group decreased rapidly on commencing skim milk, but after weaning the levels in the two groups converged, so that no significant difference could be seen.

The Human Infant: Perinatal Observations. The plasma cholesterol and triglyceride levels of the human neonate are considerably lower than in the adult. Some published values are shown in Table 1.6. The mean cholesterol values at birth lie between 62 and 95 mg/100 ml in normal infants, whilst the triglyceride values range from 32 to 69 mg/100 ml.

Few factors have been shown to affect the cholesterol level at birth. Sex may be an influence, female neonates having significantly higher cholesterol levels than males in 2 of the studies shown in the table (Barnes *et al*, 1972; Darmady *et al*, 1972). It has been suggested that infants born to diabetic mothers have higher plasma cholesterols than healthy babies; both studies to date are of small numbers. Mortimer (1964) and Pantelakis *et al* (1964a) showed that the cholesterol level tended to be higher in the infant born to a diabetic mother who had been treated with insulin throughout pregnancy, and even higher when the women were not treated with insulin (Table 1.6). Race appears to be of little importance, the New Guinean and Australian babies having similar cholesterol levels (Whyte and Yee, 1958), as did white and negro babies described by Glueck *et al* (1971). Maternal diet was also of no importance. Other factors which have been found unimportant are the maternal plasma cholesterol at the time of delivery (Kaplan and Lee, 1965; Zee, 1968; Glueck *et al*, 1971; Barnes *et al*, 1972) and the infant's birth weight (Brody and Carlson, 1962; Barnes *et al*, 1972).

The possibility that strong genetic influences may be detected at birth has received a great deal of attention, particularly in the field

TABLE 1.6

Plasma Cholesterol and Triglyceride Concentrations at Birth and During Infancy in the Human

		Plasma lipid concentration (mg/100 ml) (mean ± S.D.) (n)			
		Cord Blood	First Week	3-6 weeks	12 months
Barnes *et al* (1972)	Cholesterol	76 ± 19 (747)[1]			
	Triglyceride	52 ± 18 (747)			
Darmady *et al* (1972)	Cholesterol	78 ± 23 (302)[2]	155 ± 31 (300)	155 ± 31 (257)	191 ± 36 (273)
Glueck *et al* (1971)	Cholesterol	64 ± 19(1800)[3]			
Tsang *et al* (1974b)	Cholesterol	61 ± 11 (32)[4]			131 ± 33 (32)
	Cholesterol	136 ± 47 (12)[5]			237 ± 59 (12)
	Cholesterol	116 ± 21 (30)			164 ± 17 (30)
	Cholesterol	95 (74)			161 (74)
Zee (1968)	Cholesterol	62 ± 19 (10)	no change[6]		
	Triglyceride	32 ± 15 (10)	60[6]		
Kaplan and Lee (1965)	Cholesterol	95 ± 18 (50)	140[7]		
	Triglyceride	34 ± 14 (50)	92[7]		
Mortimer (1964)	Cholesterol	75 ± 25 (20)[8]			
	Cholesterol	105 ± 63 (13)[9]			
Pantelakis *et al* (1964a)	Cholesterol	71 ± 13 (5)[8]			
	Cholesterol	83 ± 23 (20)[9]			
	Cholesterol	110 ± 30 (11)[10]			
McKerrow (1961, 1962)	Cholesterol	77 (232)		191 ± 44 (20)[11]	
Sweeney *et al* (1961)	Cholesterol	75 ± 4 (21)*		136 ± 5 (10)*	
	Triglyceride	69 ± 5 (21)*		183 ± 25 (10)*	
Whyte and Yee (1958)	Cholesterol	70 ± 19 (25)[12]			145 ± 40 (6)
	Cholesterol	68 ± 17 (13)[13]			151 ± 37 (6)

*Mean ± S.E.M. (n)

[1]Females, 79 ± 20; males, 74 ± 18 (p <.001)

[2]Females, 81 ± 25; males, 76 ± 22 (p <.05)

Caucasion and Negro babies

[3]Normolipidaemic infants at birth with normolipidaemic parents

[4]Hypercholesterolaemic infant at birth with hypercholesterolaemic parent

[5]Hypercholesterolaemic infant at birth with normolipidaemic parents

All infants

[6]At 10 hours post partum

[7]At 3 days of age

[8]Infants born to normal mothers

[9]Infants born to diabetic mothers receiving insulin

[10]Infants born to diabetics not receiving insulin and to pre-diabetics

8. vs 10. p = 0.001

[11]Breast-fed infants only

[12]Caucasion (Australian) infants

[13]New Guinea natives

of the inheritence of Type II hypercholesterolaemia, a disease which follows best a monogenic pattern of autosomal dominance (Fredrickson and Levy, 1972). Early detection of babies inheriting Type II disease should allow prophylactic treatment to be instituted early. The significance and reliability of cord blood lipid levels as a predictor of inheritence will be discussed later. The large American series (Glueck *et al*, 1971) of 1800 births allowed an analysis of the distribution of cord cholesterol to be made. It did not conform to a single normal (Gaussian) distribution, and fitted best 2 overlapping normal distributions. Small subsets of the original data are included in Table 1.6 showing the difference in response with time of 3 groups of infants according to their initial umbilical cord blood recording and their family history of hypercholesterolaemia.

The mean cord plasma triglyceride is lower than in the adult (Table 1.6). The distribution is markedly skewed to the right (Barnes *et al*, 1972), and may be corrected to a normal distribution by expressing the values logarithmically. A similar distribution and correction was described by Brody and Carlson (1962). Recently there has been increased awareness of the value of monitoring physiological and biochemical parameters during labour and immediately after delivery. This has emphasized the influence of perinatal environmental factors upon the infant, and a recent report (Tsang *et al*, 1974a) presents a strong association between perinatal events, particularly foeto-maternal problems, and cord triglyceride levels. Further evidence for this will be presented within this thesis.

There are few systematic studies of plasma triglyceride changes with age in infancy. Zee (1968) has reported little change in the triglyceride concentration from birth until about 9 hours post partum, when there was an apparent rapid doubling in normal babies that had received neither food nor water. Plasma cholesterol did not change.

Plasma free fatty acids, low at birth, rise rapidly in the neonatal period, reaching maximal levels at 6-12 hours post partum (van Duyne and Havel, 1959). Concurrently with the increase in free fatty acid there is a reciprocal decrease in blood glucose (Chen *et al*, 1965). Administration of glucose *in vivo* will prevent the rise of free fatty acid (Novák *et al*, 1961). *In vitro* studies with neonatal adipose tissue show inhibition by glucose of free fatty acid release after 15 hours of age (Novák *et al*, 1965). The rise in plasma triglyceride 9 hours post partum may result from increased hepatic triglyceride synthesis secondary to fatty acid mobilization (Havel, 1961).

The major increase in plasma cholesterol concentration occurs in the first week and subsequent increases are relatively smaller. For instance, in the group of children studied by Darmady *et al* (1972), the cord blood cholesterol showed a doubling within the first week of life but subsequently rose only a further 13% during the first year. The plasma cholesterol concentration at one year is approximately 2 to 3 times that at birth. In the sample shown in Table 1.6 this relationship holds for the hypercholesterolaemic children as well (Tsang *et al*, 1974b).

The plasma cholesterol concentration may increase slowly during childhood and adolescence in Western Society (Table 1.7: Whyte and Yee, 1958; Hames and Greenberg, 1961; Godfrey *et al*, 1972; Friedman and Goldberg, 1973). A recent study of 613 school boys aged 11 to 18 years showed the plasma cholesterol decreased with age while plasma triglyceride increased (Hickie *et al*, 1974). Within Western Society there is no racial difference to be seen; the children in the white and non-white groups in Evans County have similar plasma cholesterol levels (Hames and Greenberg, 1961). There is a tendency for female adolescents to have slightly higher plasma cholesterol levels than males (Fredrickson *et al*, 1967; Godfrey *et al*, 1972). Of interest, is the contrast between the two populations studied by Whyte and Yee (1958): the Australian group

TABLE 1.7

Plasma Cholesterol and Triglyceride Concentrations During Childhood and Adolescence*

		6-10 years	11-15 years	16-20 years
Whyte and Yee (1958)	Cholesterol	164 ± 35 (13)	186 ± 50 (15)	185 ± 33 (18)[1]
	Cholesterol	141 ± 29 (24)	134 ± 38 (21)	122 ± 40 (20)[2]
Hames and Greenberg (1961)	Cholesterol	167 (166)	182 (198)	188 (80)[3]
	Cholesterol	166 (143)	182 (184)	186 (66)[4]
	Cholesterol	166 (85)	179 (104)	188 (37)[5]
	Cholesterol	168 (92)	187 (110)	195 (47)[6]
Wiese *et al* (1966)	Cholesterol	157 ± 27 (45)	172 ± 22 (6)[7]	
Fredrickson *et al* (1967)	Cholesterol		172 ± 34 (43)[7]	
	Triglyceride		61 ± 34 (43)[7]	
	Cholesterol		179 ± 33 (38)[8]	
	Triglyceride		73 ± 34 (38)[8]	
Lopez-S *et al* (1967)	Cholesterol			155 ± 29 (1164)
Godfrey *et al* (1972)	Cholesterol	≃160[9] (600)	≃170 (728)	≃180[10] (97)
	Cholesterol	≃165[9]	≃165[11]	
Friedman and Goldberg (1973a)	Cholesterol	≃150[12]	≃158[13]	
Court *et al* (1974)	Cholesterol		183 ± 40 (45)[14]	
	Triglyceride		73 ± 25 (45)[14]	
	Cholesterol		195 ± 47 (129)[15]	
	Triglyceride		77 ± 41 (129)[15]	
Hickie *et al* (1974)	Cholesterol			200 ± 33 (613)[16]
	Triglyceride			63 ± 42 (613)[16]
Wilmore and McNamara (1974)	Cholesterol	179 ± 27 (95)[17]		
	Triglyceride	61 ± 38 (95)		

*Plasma lipid concentration: mg/100 ml (mean ± S.D.)

[1]Australian Caucasion

[2]New Guinea natives

[3]White: male

[4]White: female

[5]Non-white: male

[6]Non-white: female

[7]Males: 0-19 years of age

[8]Females: 0-19 years of age

[9]Median

[10]Females

[11]Males

[12]7 months to 8 years of age

[13]9 years to 19 years of age

[14]Normal children: 2-17 years of age

[15]Obese children: 2-17 years of age

[16]Males: 11-18 years of age

[17]Males: 8-12 years of age

shows the increase in plasma cholesterol common to the society from which it was drawn, whereas the New Guinea group shows a steady decrease in plasma cholesterol from the age of one year (Table 1.6) to young adulthood. The diet and way of life of the two populations after weaning are vastly different: the diet of the Australian containing much cholesterol and being relatively high in fat, whilst that of the New Guineans is rich in carbohydrate and low in fat. The triglyceride levels, not shown here, also reflect this difference in eating habits. This indicates that the increase in plasma cholesterol with age seen in Western Society and the concurrent increase in arteriosclerotic disease is not an inevitable part of aging.

Lipoprotein Distribution at Birth and in Infancy. Table 1.8 shows some of the more recent published values for lipoprotein distribution at birth (part A) and in early infancy (part B). Most of these have been obtained by ultracentrifugation combined with lipoprotein-complex precipitation using polysaccharides and divalent ions (Hatch and Lees, 1968). They compare well with percentage distributions obtained by lipoprotein electrophoresis.

There is very little cholesterol present in VLDL at birth, with the bulk being distributed between LDL and HDL. The ratio between HDL and LDL in cord blood is of the order 4 to 3 in most reports apart from Glueck *et al* (1971): the LDL value quoted in that study contains VLDL plus LDL. With increased production of triglyceride 12 hours post partum, there is an increase in VLDL levels (Zee, 1968) and within the first 24 hours, LDL increases also (Abrams and Freeman, 1969). HDL has been found to increase 25% in the first week of life, and only slowly thereafter (Abrams and Freeman, 1969; Studd *et al*, 1970). Dietary cholesterol and fatty acid composition of milk formulas have been shown to influence the levels of cholesterol and lipoproteins in the infant (Table 1.9).

TABLE 1.8

Distribution of Plasma Lipoproteins and Lipids at Birth and During Infancy

		n	Plasma lipid or protein concentration (mg/100 ml)			
			VLDL	LDL	HDL	Total
A. AT BIRTH IN UMBILICAL CORD PLASMA						
Glueck *et al* (1973)[1]	Cholesterol	39	4 ± 9*	26 ± 13	36 ± 11	71 ± 15
	Triglyceride					33 ± 47
Greten/Schettler (1973)[1]	Cholesterol	1323		35 ± 12		60 ± 20
Kwiterovich *et al* (1973)[1]	Cholesterol	36	6 ± 4	31 ± 6	37 ± 8	74 ± 8
	Triglyceride					37 ± 15
Glueck *et al* (1971)[2]	Cholesterol	596	37 ± 21		32 ± 11	
Wille and Phillips (1971)[3]	Protein	12	24 ± 12	58 ± 15	81 ± 43	
Sweeney *et al* (1962)[4]	Protein	19		59.6 ± 3.1%	40.4 ± 3.1%	
	Cholesterol					75 ± 3**

*Mean ± S.D. apart from
**Mean ± S.E.M.

Method of Separation:

[1]Ultracentrifugation and precipitation

[2]Precipitation

[3]Agarose gel electrophoresis

[4]Paper electrophoresis

Continued

TABLE 1.8

(Continued)

		n	Plasma lipid or protein concentration (mg/100 ml)			
			VLDL	LDL	HDL	Total
B. DURING INFANCY						
Greten/Schettler (1973)[1]	Cholesterol	126		113 ± 33		172 ± 34
Fredrickson *et al* (1967)	Cholesterol[2]	43	9 ± 7	108 ± 33	49 ± 11	61 ± 34
	Triglyceride					179 ± 33
	Cholesterol[3]	38	11 ± 8	108 ± 10	53 ± 12	73 ± 34
	Triglyceride					
Sweeney *et al* (1962)[4]	Protein	9		69.4 ± 4.9%	30.6 ± 4.9%	
	Cholesterol					135 ± 5**

*Mean ± S.D. apart from
**Mean ± S.E.M.

Age:

[1]1 year of age

[2]Males: 0-19 years of age

[3]Females: 0-19 years of age

[4]6-8 weeks of age

2. Diet and Plasma Cholesterol Concentration During the First Year of Life Numerous short term studies have been undertaken in the young infant demonstrating the effect on plasma cholesterol levels of different milks. Some of these studies are summarized in Table 1.9. They show mainly the effect of fatty acid composition of different formulae. The studies are not strictly comparable in terms of either the age of the children or the composition of formulae.

Diet is a very important determinant of the plasma cholesterol level during infancy. Comparing groups at any age during the first year, there is a marked difference in the levels found in infants consuming evaporated cow's milk products and milk products to which vegetable oils have been added. The plasma cholesterol level is generally reciprocally related to the level of linoleic acid in the milk, i.e., the higher the level of linoleic acid, and the higher the ratio of polyunsaturated to saturated fatty acids, the lower the plasma cholesterol of the group. From most of the studies in Table 1.9, it is not possible to draw any conclusions about the relative importance of cholesterol in the products. As pointed out by Tsang *et al* (1974b) it is often very difficult to separate the effect of cholesterol and fatty acid in analyzing dietary effects, since diets low in cholesterol generally also contain vegetable oils.

Although human milk has a higher linoleic acid content than cow's milk, the majority of studies comparing the two demonstrate either very similar plasma cholesterol levels in the infants, or even a higher level in the breast-fed children. One exception to this (György *et al*, 1963) is a study in premature infants in whom human milk led to lower plasma cholesterol values than feeding with evaporated cow's milk. However, the former infants had very poor weight gain and were suffering from hypoproteinaemia. Children being fed pooled breast milk to which casein had been added had plasma levels very similar to those taking cow's milk and evaporated milk and gaining weight normally.

TABLE 1.9

The Effect of Diet on Plasma Cholesterol Levels During Infancy

	Milk Formula	Linoleic Acid*	Plasma Concentration (mg/100 ml)		
Sweeney *et al* (1961, 1962)			*6-8 weeks*[1]		
	Evaporated	1.2%	136 ± 5 (10)[2]		
	Soya/Coconut	39.2%	89 ± 5 (10)		
	Milk/Vegetable Oil	30%	88 ± 4 (9)		
Lowe *et al* (1964)			*8 weeks*	*16 weeks*	
	Milk + Butterfat[3]		142 ± 23[5]	143 ± 29	
	Milk + Corn Oil[4]		117 ± 31	114 ± 35	
Fomon and Bartels (1960)				*1 week-6 months*[5]	
	Human Milk	8%		172 ± 42 (21)	
	Cow's Milk	4%		156 ± 56 (8)	
	Milk/Vegetable Oil	30%		144 ± 43 (30)	
	Soya/Vegetable Oil	46%		108 ± 27 (5)	
György *et al* (1963)[6]			*3-5 weeks*	*9-13 weeks*	*21-25 weeks*
	Lactum[7]		144 ± 4 (95)[2]	145 ± 3 (67)	163 ± 7 (41)
	S.M.A.[7]		104 ± 3 (77)	112 ± 4 (72)	129 ± 4 (42)
	Sobee[7]		99 ± 5 (25)	122 ± 6 (26)[8]	139 ± 8 (14)
	Human Milk		106 ± 7 (8)	117 ± 6 (12)	
	Cow's Milk		111 ± 3	119 ± 4	148 ± 6
Woodruff *et al* (1964)			*6 weeks*	*3 months*	*6 months* *12 months*
	Human Milk	10.5%	159 ± 9 (22)[2]	145 (5)	163 (2) 147 (7)
	Evaporated Milk	2.6%	156 ± 5 (21)	155 ± 7 (19)	146 ± 20 (13) 134 ± 11 (9)
Darmady *et al* (1972)			*6 weeks*	*4 months*	
	S.M.A.[7]	≃20%	129 ± 27 (19)[5]	149 ± 30 (31)	
	Human Milk		175 ± 29 (24)	196 ± 30 (15)	
	Cow's Milk		158 ± 26 (148)	190 ± 33 (199)	

Continued

TABLE 1.9

(Continued)

	Milk Formula	Linoleic Acid*	Plasma Concentration (mg/100 ml)	
		Birth		*12 months*
Tsang *et al* (1974b)	Low Cholesterol[9]	66 ± 44 (10)[5]		135 ± 19
		138 ± 37 (5)		161 ± 27
	Moderate/High Cholesterol[10]	61 ± 11 (32)		131 ± 33
		136 ± 47 (12)		233 ± 59

*When concentration included in the report.

[1]Age of infants

[2]Mean ± S.E.M. (n)

[3]Butterfat = 51% of calories

[4]Corn Oil = 52% of calories

[5]Mean ± S.D. (n)

[6]Premature infants

[7]Commercial milk preparations

[8]Poor weight gain and hypoproteinaemic

[9]100-300 mg cholesterol/day and P/S ≃ 1.5:1

[10]500-1000 mg cholesterol/day and P/S ≃ 0.4:1

The importance of diet in determining plasma cholesterol levels during infancy is illustrated by the results of Tsang and his colleagues (1974b) (Table 1.9). This compares infants who were normocholesterolaemic and hypercholesterolaemic at birth. At 12 months of age both groups had similar cholesterol levels when on a low cholesterol, high P/S ratio diet. Only when challenged with a high cholesterol, low P/S ratio diet did the genetic trait show.

Reservations on the Early Use of Diet. The human infant responds rapidly to changes in the saturation of dietary fatty acids. Pomeranze *et al* (1958) studied 3 infants for 15 to 31 weeks, alternating feeding periods of evaporated milk and corn oil-supplemented milk. The plasma cholesterol levels rose and fell. They noted also that infants placed on a soy milk preparation from birth did not show the expected increase in plasma cholesterol concentration after birth. Concern has been expressed regarding long term exposure to low cholesterol or high P/S ratio diets. It has been feared by some that inadequate exposure to cholesterol in early infancy or childhood may prevent the child from responding appropriately to dietary cholesterol later in life (Fomon, 1971). The argument is, that low cholesterol diets early in life would be detrimental and achieve the opposite of that desired, namely hypercholesterolaemia later in life. This has received support from some animal experimentation conducted in rats and pigs. Kubát (1966) weaned rats at 18 and 30 days with a high fat, low cholesterol diet. Serum cholesterol levels at 10 months revealed no difference, but following exposure to a cholesterol-rich diet for $2^{1}/2$ months, the serum cholesterol was higher in the group weaned early. Further experiments in rats by Reiser and Sidelman (1972) manipulated the maternal diet to raise the cholesterol concentration of the rat's milk (from a "normal" 24 mg/100 ml to 40 mg/100 ml). Suckling, with access to the maternal feed, was allowed for 30 days post partum, following which a low cholesterol, 10%

stripped lard diet was instituted. At 60 days, the animals were challenged with 0.5% cholesterol in lard. Among the male rats, the resultant serum cholesterol was inversely related to their dam's milk cholesterol concentration. The authors attributed the results to the presence of dietary cholesterol from early in life, which primed the negative feedback mechanism. However there is no data about the fatty acid compositions of the milks, and the young had free access to the maternal diet. They considered the presence of another inhibitor of cholesterol synthesis in rat milk most unlikely. However, such an agent does appear to exist, in both rat milk, cow's milk and human milk (McNamara *et al*, 1972; Boguslawski and Wróbel, 1974). Evidence for a possible need for early exposure to cholesterol comes from another study by Reiser (1971) in which small groups of pigs were given low (1.4 mg/100 ml) or high (115 mg/100 ml) cholesterol diets whilst suckling. Subsequent challenge with high cholesterol diets resulted in higher plasma cholesterol levels in those previously fed small amounts of cholesterol.

Considering the human infant, the response as seen in plasma cholesterol levels at 12 months of age does not seem to be affected by the dietary intake of cholesterol during the first 6 months of life (Glueck *et al*, 1972). The response was similar among normocholesterolaemic and hypercholesterolaemic infants. However, long term follow-up will be necessary to determine with certainty the long term effect of low cholesterol, high P/S ratio diets in ensuring an adequate feedback mechanism to dietary cholesterol in adulthood. In view of the widespread use of commercial milk formulae containing lower amounts of cholesterol and higher P/S ratios than are present in human or cow's milk, this subject should be kept under review. A preliminary report states that no long term differences in serum cholesterol could be detected in adolescents aged 15-19 years who had been breast-fed or artificially-fed on low cholesterol milk formulae (Friedman and Goldberg, 1973b). This

study did however find that the breast-fed children had higher cholesterol levels at 2 years of age, 20 months after having been weaned on a skim milk, "generally low" cholesterol and saturated fat diet.

Another area of concern associated with the feeding of artificial linoleate enriched diets has been the effect on growth, development and maturation of the central nervous system. In the human infant, the brain is relatively immature at birth; further growth and myelination occurs at a high rate in the first few months of life. Cholesterol is an essential component of membrane structure. Animal experimentation however has demonstrated that whilst preformed, and therefore dietary, cholesterol can be incorporated into brain sterols, this occurs only at relatively slow rates. Most of the CNS cholesterol is derived from *in situ* biosynthesis, an efficient precursor for which is glucose. Specific examples of this work include that by Morris and Chaikoff (1961) in neonatal rats, to whose mothers labelled-cholesterol had been administered pre- and post-partum, and by Chevallier (1965), who demonstrated minimal contributions from exogenous cholesterol to the brain in the neonate. Numerous other studies are discussed in relation to brain cholesterol synthesis and turnover in Kabara's review (1965).

Changes in the total fat content or fat composition of milk have also been raised as areas for caution in infant nutrition. The total fat requirement in the infant diet has not been defined (Schubert, 1973). In human milk some 50% of the calories are derived from fat. The anxiety regarding reduction of total fat in infancy is again based on animal experimentation. Rats, weaned early and therefore receiving fewer calories from fat, suffer from defects in conditioned reflexes and learning behaviour as adults (Novakova, 1966). No comparable human data is available. The composition of fat in terms of individual fatty acids is important. Linoleic acid deficiency is well documented in human infants and a minimal requirement equivalent to 1 percent of the total calories, and optimally 4.5 percent, has been determined (Combes *et al*,

1962). These values are comparable to the levels of linoleic acid in human milk. The desirability of increasing the polyunsaturated fatty acid content of foods in infancy has also been questioned. Human infants, particularly premature infants, have low levels of vitamin E, due to limited placental transfer (Wright *et al*, 1951), and it has been shown that polyunsaturated fatty acid rich formulae increase the requirements for vitamin E in the premature baby (Hassan *et al*, 1966). Symptoms do not appear in healthy full-term infants. Supplementation of the commercially available formulae with α-tocopherol has been instituted. A short-term study of children aged 5-21 years, with familial type II hypercholesterolaemia, during which treatment with diet and cholestyramine was instituted, showed that neither was an immediate threat to maintenance of adequate vitamen E levels in the blood (Glueck *et al*, 1974).

In defence of the low cholesterol, higher P/S ratio diet of infancy, it must be said that to date there is no evidence of any harmful effects. In terms of positive benefit to a population at risk the potential has yet to be proven. However, in all studies to date, infants using commercial formulae grow well and develop normally during infancy and childhood (Fomon and Bartels, 1960; Sweeney *et al*, 1961; Fomon *et al*, 1970). Similarly, children participating in a long term trial of treatment at the University of Arizona have shown no distinguishing features from the remainder of the population in parameters of growth and development or illness (Goldberg and Friedman, 1973).

D. THE SECRETION OF MILK

The following section on milk secretion will describe the functions of the mammary gland. This includes derivation of the substrates from plasma, such as fatty acids, the processes by which the gland concentrates the substrates and converts them into products such as triglycerides, and finally the mechanisms for excreting the products from the cell into the alveolus. Emphasis will be placed on the process

of fat secretion, fat being the constituent of major interest in the dietary studies to be discussed later in the thesis.

Lactation may be divided into two physiological entities: the first, lactogenesis or the initiation of milk secretion, and the second, gelactopoiesis, which is the maintenance of milk secretion. The type of milk produced during the periods differs quite markedly, colostrum being secreted within the first 5-6 days post partum in the human, with a transitional product preceding mature milk. The interplay of contributing factors, particularly hormonal, that occur pre-partum and post-partum and which result in milk secretion have been summarized by Baldwin (1969) in terms of 3 interrelated events which occur almost simultaneously. As he sees it, there is

(1) the formation of functionally differentiated secretory cells,

(2) the development of characteristic enzyme complements, which have the ability to synthesize milk components, and

(3) the regulation of the capacity for milk synthesis by substrate availability, hormonal interaction and demand.

1. Cellular Morphology of the Alveolar Cells and the Secretion of Milk

The alveolar cells are variable in size and shape, depending upon the stage of secretion (Richardson, 1947; Folley, 1952). The changing morphology is related to the two major stages of milk secretion. Emptying of the gland is dependent upon either suckling or milking, and this defines both the beginning and the end of a milk secretion cycle. At the beginning, the secretory phase, the cells are tall and columnar, with the alveolar epithelium folded into the lumen. As milk collects in the alveoli, the cells become stretched.

During the secretory phase, the cell nucleus moves away from the base of the cell and striations appear (Grynfellt, 1936). These have been likened to the ergastoplasmic filaments of secretory cells as described by Limon (1902) and give positive cytochemical reactions of RNA

(Verne, 1951; Vendrely, 1951). The nucleus itself changes also, with an increase in the number of vesicles and movement of its chromatin to the periphery (Bourne, 1952).

The formation of lipid inclusions in the basal part of the alveolar cells and their subsequent coalescence was described as early as the mid-nineteenth century, by Koelliker (1852) and Virchow (1858). The association of the lipid inclusions with a cellular organelle, the mitochondrion, was first noted by Hoven (1911). Lipid droplets have been detected in alveolar cells early in pregnancy in rabbits (Uggeri, 1939). In the rat, sudanophil droplets in the alveolar cells and lumen immediately preceded the secretion of colostrum (Dempsey *et al*, 1947).

In the secretion phase of mature milk, migration of lipid droplets occurs from base to apex in the alveolar cells of the rat (Dempsey *et al*, 1947). The synthesis of fat is believed to take place in the rough endoplasmic reticulum of the cell. For instance, Stein and Stein (1967) using autoradiography combined with electromicroscopy, demonstrated that fatty acid esterification into glycerides takes place here, and *in situ* aggregation of lipid then occurs with the appearance of fat droplets.

The secretion phase is succeeded by one of cellular excretion, which results in distension of the alveoli. Grynfellt (1936) described colostral excretion as follows:

> a thickening of the apical membrane of the alveolar cell preceeds a change in shape of the membrane, firstly hemispherical convex to the lumen and then clubshaped; contained within the super-apical club, the collection of material, lipid and/or protein. The base of the club finally breaks, and the club and its contents become part of the luminal contents.

Electron microscopy has been used to study milk secretion and excretion. Bargmann and Knoop (1959) and Hollmann (1959), using rat and mouse mammary tissue, described the excretion of lipid inclusions. The globules leave the cell in the form of fat corpuscles surrounded by a cytoplasmic membrane, double in outline and 170-200 Å thick. Expulsion

occurs by a process of constriction and the cell cytoplasm is never exposed. This process has also been observed in bovine mammary cells in culture (Kinsella and McCarthy, 1968). As the constriction narrows, varying amounts of cytoplasm may be entrapped (Stein and Stein, 1967; Helminen and Ericsson, 1968). It has been estimated that 1-5% of fat globules in milk may be secreted with cytoplasm attached (Wooding *et al* (1970).

The milk-fat globule membrane is derived from the plasmalemma of the alveolar cell (Patton and Fowkes, 1967), with subsequent rearrangement of the membrane constituents to increase its stability (Keenen *et al*, 1970). A thick layer of adsorbed or bound neutral lipid apparently derived from the fat globule accumulates on the apical plasma membrane as the fat globule is enveloped (Keenen *et al*, 1971).

Milk protein appears as finely granular material within the Golgi apparatus of the cell, having been synthesized in the rough endoplasmic reticulum. From here the proteins move towards the apex of the cell, enclosed in vacuoles along with other cellular products such as lactose (Keenen *et al*, 1970). The vacuoles come to rest just below and in contact with the plasmalemma, and finally open outwards (Helminen and Ericsson, 1968). These vacuolar membranes may help replace plasmalemma lost during lipid excretion.

2. Composition of Milk The composition of milk and its variation in different species of mammal, particularly in terms of its nutritive value, has frequently been used as a means of assessing lactation itself. The following section will present, mainly in tabular form, some of the basic components of human milk, compared with the concentration in cow's milk. Many variations exist, not only inter-species, but intra-species, with individuals producing milk of widely differing composition. Factors which may alter both the quality and quantity of milk secreted have been enumerated by Macy and Kelly (1961);

(1) the stage of lactation,

(2) inherent biochemical and physiological differences, as well as psychological contributions,

(3) the amount and kind of food consumed,

(4) environmental conditions,

(5) disease,

(6) the size and structure of the mammary gland,

(7) heredity, and

(8) the method of sampling.

Physically milk is a complex system, at its time of secretion consisting of two liquid phases, fat and water, between which are divided over 40 different compounds. Table 1.10 shows these constituents divided into three main groups, with average values of their major components. Dissolved in the fat, within the emulsified part of the milk, or held at the fat globule surface, are the compounds of Group A, for instance phospholipids, sterols and fat-soluble vitamins. The aqueous phase may be subdivided into two categories, once again defined in physical terms, for it carries in solution compounds such as lactose and in colloidal suspension proteins, whilst the mineral elements are divided between the two.

Milk Fat. The bulk of lipid in milk is present in fat globules 2-3μ in diameter, each globule being surrounded by a membrane whose hydrophilic surface is responsible for maintaining it in an emulsified state, and which is considered to offer the contained glycerides some protection against lipase activity (Ling, Kon and Porter, 1961). Rearrangement may occur in the post-secreted membrane with aging as seen in ultrastructural studies (Bargmann and Knoop, 1959), particularly apparent loss of globule membrane into the milk plasma (Wooding, 1971). At the time of secretion, the membrane has the classical trilaminar structure of the plasma membrane, approximately 90 Å thick, of lipid/protein (Patton, 1973). Little is known to date of the molecular architecture of the

TABLE 1.10

Constituents of Human Colostrum and Mature Milk Compared With Cow's Milk

(values per 100 ml of whole milk)

	Human Milk				Cow's Milk
	Colostrum		Mature		Mature
	$\bar{x}$*	range**	$\bar{x}$	range	$\bar{x}$
A. FAT					
Total, inc. Triglyceride, g	2.9	2.5-3.2	3.8	1.3 -8.3	3.7
Fat Soluble Compounds					
Cholesterol, mg	27		20		14
Phospholipids, mg (phosphorus)	2		4		4
Caretenoids, µg	112		27		38
Vitamins[1]					
B. NON-FAT SOLIDS					
Nitrogenous					
Protein - Total, g	2.3	1.5-6.8	1.1	0.7 -2.0	
Casein, g	1.2		0.4		2.8
Albumin			0.2		0.2
β Lactoglobulin, g			0.3	0.14-0.60	0.4
α Lactalbumin, g			0.03		0.04
Serum Albumin, g	0.3				
Globulin			0.01		0.1
Serum Globulin, g	0.1				
Enzymes[2]			42	17-60	21
Non-Protein[3] - Total, mg					
Non-Nitrogenous					
Lactose,[4] g	5.3		7.0	5.0 -9.2	4.8
Vitamins					

Continued

TABLE 1.10

(Continued)

	Human Milk				Cow's Milk
	Colostrum		Mature		Mature
	$\bar{x}$*	range**	$\bar{x}$	range	$\bar{x}$
B. NON-FAT SOLIDS (Continued)					
Non-Nitrogenous Minerals					
Calcium, mg	31	24-66	33	17-61	125
Potassium, mg	74	66-87	55	37-64	138
Sodium, mg	48	26-136	15	6-44	58
Chlorine, mg	91	44-101	43	9-73	103
Trace Elements[5]					
C. WATER					
Dissolved CO_2, O_2, N_2					

*Mean values taken from Table III, p. 275-277, Macy and Kelly (1961).
**Ranges taken from Table I, p. 268-269, Macy and Kelly (1961).

[1]Fat-Soluble Vitamins - A, D, E, K.

[2]Enzymes - many have been identified, e.g. 5'-nucleotidase (Kobylka and Carraway, 1972).

[3]Non-Protein Nitrogen - creatine, creatinine, urea, uric acid.

[4]Water-Soluble Vitamins - e.g. Ascorbic acid, Biotin, B_6, B_{12}, Folic acid, Nicotinic acid, Riboflavin, Thiamine.

[5]Trace Elements - e.g. Iron, Copper, Zinc, Iodine, Fluorine.

membrane, but it is apparent that many of the marker enzymes associated with the plasma membrane are to be found on the milk fat globule membrane, for instance 5'-nucleotidase (Kobylka and Carraway, 1972).

Milk cholesterol and phospholipid are important structural components of membrane. Partition of these chemicals in milk has indicated in the cow and goat, that some 60% of the phospholipid (Patton and Keenan, 1971) and 80-85% of the cholesterol (Patton, 1973) is associated with the globule, the remainder occurring in skim milk.

The major milk fat is triglyceride. The major fatty acids found in human milk are shown in Table 1.11. As many factors are known to modify milk fat composition, the figures are only approximations. The relative proportions of saturated and unsaturated fatty acids impart many of the characteristic physical properties of milk fat, such as hardness and melting point, which may be important in the dairy industry. Human milk fat has only traces of fatty acids shorter than decanoic, whilst the presence of significant amounts of butyric acid is characteristic of ruminant fat. The unsaturated fatty acids are divided according to the number of double bands present, into monoethenoid, the main representative being oleic acid, and diethenoid. In human milk, a considerable portion of the major diethenoid acid, linoleic, remains in the *cis-cis* form, characteristic of the form derived from vegetable material, and one of the essential fatty acids for human nutrition. Ruminant milk contains a lower content of linoleic acid, and some of this is in the *trans-trans* and *cis-trans* forms, probably as a result of ruminal isomerization (Scott *et al*, 1959).

3. Synthesis of Milk Fat

Fatty Acids and Triglyceride. The arterio-venous difference of total fatty acids across the perfused cow udder is sufficient to account for all milk lipid as described by Graham *et al* (1936). This observation has been confirmed for both cow and goat by other workers using similar

TABLE 1.11

The Fatty Acid Composition of Milk

Fatty Acid Composition (g/100 g fat)	Human Milk		Cow's Milk
	Colostrum	Mature	Mature
SATURATED			
Butyric	0.2	0.4	3.1
Caproic	0.1	0.1	1.0
Caprylic	0.4	0.3	1.2
Capric	2.2	1.7	2.6
Lauric	1.8	5.8	2.2
Myristic	3.8	8.6	10.5
Palmitic	26.2	22.6	26.3
Stearic	8.8	7.7	13.2
Arachidic	3.8	1.0	1.2
UNSATURATED			
Decanoic	0.2	0.1	0.2
Dodecanoic	0.1	0.1	0.2
Tetradecanoic	0.2	0.6	1.1
Hexadecanoic	2.4	2.9	3.1
Oleic	36.6	36.4	32.2
Octadecadienoic	6.8	8.3	1.6
Arachidonic	1.7	0.8	1.0
C_{20}-C_{22}	10.2	4.2	1.0

techniques (e.g. Maynard *et al*, 1938; Shaw *et al*, 1940). This was the culmination of many years during which attempts were made to demonstrate a relationship between blood and milk lipids. However, whilst the quantity was now accounted for, the quality in terms of fatty acid composition was not. That fatty acids derived from plasma will appear unchanged in milk was demonstrated using elaidic acid (McConnell and Sinclair, 1937) and since then many have shown the ready passage of labelled free fatty acid into milk (e.g. Glascock *et al*, 1956; Annison *et al*, 1957; Bickerstaffe, 1972).

The high respiratory quotient (R_Q) of lactating udder pointed to the synthesis of fat from carbohydrate (Graham *et al*, 1938) and Rittenberg and Bloch (1944, 1945) were subsequently able to demonstrate synthesis of fatty acids from 1-^{13}C acetate in the mammary glands of mice. The incorporation of acetate-carbon into the mammary gland of pregnant rabbits (Popják and Beeckmans, 1950) was soon followed by the demonstration of labelled short chain fatty acids appearing in goat's milk after administration of 1-^{14}C acetate (Popják, French and Folley, 1951). *In vitro* studies have demonstrated that both ruminant and rodent mammary tissues synthesize fatty acids via the malonyl-CoA pathway, using NADPH, most of which is supplied from the pentose cycle (Glock and McLean, 1958; Jones, 1969). The difference in substrate utilization in different animals is well documented. The rat is able to incorporate glucose-carbon into fatty acids (Folley and McNaught, 1961; Katz and Wals, 1972), whilst the ruminant does not, relying on plasma acetate and β-hydroxybutyrate as its prime sources (Folley and McNaught, 1961; Wood *et al*, 1965; Smith, 1971). These differences result in characteristic fatty acid patterns, ruminant milk fat having a high proportion of short chain acids, i.e. C_4-C_{14} (Garton, 1963).

The mammary gland, then, has the capacity to extract fatty acid from the blood and to synthesize fatty acid from small carbon precursors.

What is the relative importance of the two mechanisms? Linzell (1968) studied the contributions of the two systems in the goat and found that the animal removed significant quantities of acetate, β-hydroxybutyrate, free fatty acid and triglyceride from the chylomicron and LDL fractions of plasma, as well as some free glycerol. All the milk fatty acid up to and including C_{14} (myristate), plus some 50% of C_{16} (palmitate) was exclusively synthesized from acetate and β-hydroxybutyrate. This accounted for 40% by weight of the total milk fatty acids. The remainder, that is mainly C_{18} (stearate and oleate), was taken up directly from plasma free fatty acids and triglyceride. This is in agreement with the findings of Popják *et al* (1951b) who found that the goat was unable to synthesize fatty acids beyond C_{16}.

The mammary gland extracts from plasma both dietary lipid and lipid synthesized elsewhere in the body. That non-physiological fatty acids may find their way into milk lipids was demonstrated by the elaidic acid study mentioned earlier (McConnell and Sinclair, 1937). In the same year, Aylward *et al* (1937) found iodized-fat in milk after administering it in food. Using tracer doses of labelled stearic acid in the diet, Glascock *et al* (1956) achieved recoveries of up to 60% in cow's milk and 45% in goat's milk, radioactivity being detected as early as 4 hours and reaching maximal activity within 23 hours. Thereafter the specific activity decreased, with a half-life of some 15 days. It was calculated that a maximum of 27% of the milk fat was derived directly from dietary fat in these studies provided that labelled stearic acid was representative of other dietary fatty acids.

The influence of diet upon the fatty acid composition of milk has received considerable attention. The first attempt to alter human milk fat by dietary means was by Thiemich in 1898, during studies demonstrating changes in the iodine number of milk fat after feeding mothers unsaturated fat. Söderhelm (1953), using corn and fish oils, was able to increase the diene and higher polyene fatty acid contents

of milk. In a study designed primarily to investigate fat metabolism, Insull *et al* (1959) markedly influenced the fatty acid profile in human milk by dietary change. With a 40% fat (corn oil) diet, there was a 5-fold increase in linoleate and linolenate compared to a diet in which the fat was more saturated. With carbohydrate-rich diets, whether given in hypocaloric or hypercaloric amounts, the proportion of long chain acids (C_{18}) decreased and shorter chain fatty acids (C_{12} and C_{14}) increased. Both Insull *et al* (1959) and Read *et al* (1965) who demonstrated similar effects with high carbohydrate, low fat diets, considered that the C_{12} plus C_{14} fatty acids reflected intramammary synthesis of fat. Changes in the fatty acid pattern of milk may also be achieved in animals by dietary manipulation, particularly in regard to increasing the poly-unsaturated fatty acid content of milk (Parry *et al*, 1963; Cook *et al*, 1970).

In discussing the relative contributions to milk lipid from dietary and endogenous sources, it was stated that in the goat, significant amounts of lipid precursor are derived from the chylomicron and LDL fractions of plasma (Linzell, 1968). This transfer is brought about by lipoprotein-lipase, which increases in mammary tissue during lactation (McBride and Korn, 1963; Robinson, 1963; Hamosh *et al*, 1970). The release of lipoprotein lipase into venous plasma has been demonstrated in the goat (Barry *et al*, 1963), with resultant hydrolysis of chylomicrons (West *et al*, 1967). With the electron microscope, Schoefl and French (1968) demonstrated an initial adhesion of chylomicrons to the endothelium of capillaries in mouse mammary tissue, followed by their engulfment. It has been estimated that up to 50% of chyle leaving the intestine of the goat may be extracted by the mammary gland (Lascelles *et al*, 1964).

The site of esterification of fatty acids is the rough endoplasmic reticulum of mammary alveolar cells (Stein and Stein, 1967). More

recently it has been demonstrated that a component within the cytosol of homogenized pig or goat mammary tissue will stimulate synthesis (Bickerstaffe and Annison, 1971).

In vitro tissue slice and perfusion experiments have indicated that the mammary gland itself is not the site of glycerol production (Balmain *et al*, 1953; Dimant *et al*, 1953; Lauryssens *et al*, 1957). In several species, the glycerol moiety as well as the fatty acids released by triglyceride hydrolysis is incorporated into milk triglyceride (McBride and Korn, 1964c; West *et al*, 1972). Although glycerokinase is present in guinea pig mammary tissue (McBride and Korn, 1964a), the 2-monoglyceride derived by hydrolysis of plasma triglyceride is probably the primary form in which the glyceryl moiety is taken up by the alveolar cells, with glycerol itself being released into the bloodstream (Scow *et al*, 1973). In the goat, the incorporation of glycerol into milk triglyceride is negligible (Pynadath and Kumar, 1964; West *et al*, 1972). Two potential pathways for triglyceride synthesis have been demonstrated in the mammary gland *in vitro*. The first is the 2-monoglyceride pathway, which was shown to exist in guinea pig mammary tissue by McBride and Korn (1964b). This requires prior activation of the fatty acid with adenosine triphosphate and coenzyme A, and is similar to that in intestinal mucosa (Senior, 1964). However, unequivocal evidence for this pathway has not been forthcoming (Dimick *et al*, 1966; Patton, 1973; Scow *et al*, 1973). The second pathway is that utilizing α-glycerophosphate (Kennedy, 1957), which is generated by glycolysis or the action of glycerol-kinase, which is present in mammary tissue (McBride and Korn, 1964a). It is acetylated with 2 moles of fatty acid to give 1,2-diacylphosphatidic acid. This, in turn, is converted to a diglyceride and finally acylation with a further mole of fatty acid results in triglyceride. This system has been demonstrated *in vitro* (Pynadath and Kumar, 1964; Kuhn, 1967), but until recently there has

been considerable difficulty in identifying the intermediate phosphatidic acid in the gland of the intact animal. Preliminary results from Patton (1973) report recent success.

Cholesterol. As with other components of milk, there are two possible sources of cholesterol, the plasma and the gland. Cholesterogenesis in the mammary gland has been established with tissue slices, perfusion experiments and whole body studies.

In slices of lactating rat mammary gland, acetate and mevalonate are incorporated into digitonin-precipitable sterols (Clarenberg and Chaikoff, 1966). Popják and his colleagues demonstrated this in the whole animal (Popják and Beeckmans, 1950; Popják *et al*, 1951), and the perfused udder (Cowie *et al*, 1951). Incorporation of precursors into sterols occurred rapidly following injection of label into the lactating goat (Popják *et al*, 1951), but since the cholesterol specific activity in milk and in plasma were not the same, the results were interpreted as showing cholesterol synthesis within the gland. Injecting labelled mevalonate into the lactating rat gives rise to labelled cholesterol and also triglyceride in milk, maximal activity being detected within 10 hours of injection (Patton, 1973).

The contribution of plasma cholesterol to milk has been partly resolved by measuring the influx of dietary ^{14}C-cholesterol into milk. In the rat, Chevallier (1964) found that some 80% of milk cholesterol was derived from plasma, and similar figures were published by Clarenberg and Chaikoff (1966), (73%), and by Easter (1971), (70%). The rats in Chevallier's experiments were maintained on diets containing 0.035% cholesterol, those of Clarenberg *et al* and Easter on 0.05% and 0.06% cholesterol respectively. Connor and Lin (1967) have examined the situation in lactating rabbits and guinea pigs. Labelled cholesterol appeared rapidly in milk following administration, becoming maximal within 2-5 days of a single dose. In the guinea pigs, a mean value of 35%

of the milk cholesterol was derived from plasma, whilst in the rabbit, it was mostly derived from plasma. Cholesterol may accumulate within the rabbit mammary gland prior to parturition because in animals to whom labelled cholesterol was fed during gestation, the specific activity of the milk on days 1 and 2 after parturition was higher than that in plasma at the time of parturition.

The question arises whether dietary cholesterol can influence the milk cholesterol concentration. Clarenberg and Chaikoff (1966) whilst studying the origin of rat milk cholesterol, showed that on a 0.05% cholesterol diet, 11% of milk cholesterol was derived from the diet at isotopic equilibrium, with mammary gland and milk attaining similar specific activities. In the guinea pig, the cholesterol content of milk responds to changes in dietary cholesterol, an increase of 0.1-0.5% cholesterol in the diet resulting in a 2-3 fold increase in milk cholesterol, the relative contribution from plasma being increased 2 fold, i.e. from 35% on a cholesterol-free diet to 70% on the cholesterol-containing diet (Connor and Lin, 1967). Reiser and Sidelman (1972) increased rat milk cholesterol by altering the dietary fat content, the P/S ratio or the cholesterol content.

To elucidate the way in which cholesterol is secreted into milk, it is necessary to examine its distribution within various milk fractions. Cholesterol is a major component of cell membranes, including that of the milk fat globule. Cholesterol comprises 5.2% of the membrane lipid of the bovine globule (Brunner, 1969). Milk from the cow and the goat has 80-85% of the cholesterol associated with the fat globules (Patton and Keenan, 1971; Patton, 1973). The further subdivision of this cholesterol into membrane and globule core poses technical and artefactual problems, with possible redistribution of the cholesterol during attempts at fractionation. The remaining 15-20% of milk cholesterol is found in the "skim milk" phase after removal of the fat globules. Some, if not all,

of the cholesterol in this fraction is undoubtedly associated with the membranes found in the fraction, some of which are derived from microvilli shed from the alveolar cell surface (Stewart *et al*, 1972), and some is shed from the fat globule membrane by vesiculation (Wooding, 1971). Cholesterol is present in both its free and esterified forms, but the ratios vary widely in the literature (e.g. Huang and Kuksis, 1967). There is a very rapid turnover of fatty acid in the cholesterol ester of whole milk (Keenan and Patton, 1970), and at least a portion arises from the plasma membrane of the secreting cell (Keenan and Patton, 1970).

Easter (1971, 1973) has attempted a model system of cholesterol excretion into milk. Following alimentary administration of labelled cholesterol, 25-50 hours (Easter, 1971) or 2-5 days (Connor and Lin, 1967) is required before maximal specific activity is attained in milk. Easter (1971) demonstrated a good precursor-product relationship between serum and milk cholesterol with an estimated turnover time of 17-20 hours for the movement from serum to milk. On this basis cholesterol is incorporated indirectly into milk, being incorporated into the alveolar cell membranes in a regenerative capacity, the cell membranes of the endoplasmic reticulum and Golgi apparatus being directly involved in the ferrying of protein and lactose to the luminal surface, and then themselves being utilized in the milk fat globule membrane (Section 1). Subsequent confirmation of this was obtained by preferential labelling of the skim milk membrane material from radioactive plasma cholesterol, the specific activity of the former exceeding that of the total plasma (Easter, 1973). On the other hand, use of the precursor mevalonate results in label being detected in the cholesterol of the milk fat globule fraction, with maximal specific activity occurring within 10 hours. This implied direct incorporation of endogenously synthesized cholesterol into the milk fat globule itself during its secretion from the alveolar cell. In summary

then, Easter has hypothesized that plasma cholesterol is initially incorporated into alveolar membranes via cell membrane regeneration and later excreted into milk indirectly, whilst cholesterol which is synthesized within the gland is an immediate component of the milk fat globule itself.

Phospholipid. The third lipid component of milk to be considered is phospholipid. It comprises 20.4% of bovine milk fat globule membranes (Brunner, 1969). Differences in fatty acid composition of milk phospholipids are attributed to nutritional, environmental and genetic factors (Jensen, 1973). Fractionation of milk indicates that 42% of lipid phosphorus is in the skim milk, whilst 58% is in the milk fat globule (Patton and Keenan, 1971). In most cases the phospholipids from both sources are similar, as are the fatty acid profiles, with some differences in the partitioning of cerebrosides. This implies differences in origin of the two fractions.

The source of milk phospholipids is predominantly *de novo* synthesis within the gland. Labelled phospholipid and inorganic phosphorus is detected in rat milk maximally 6 hours after intravenous radioactive inorganic phosphate is administered (Easter *et al*, 1971). In the goat the label appears for the first 12 hours as inorganic phosphate with progressively more radioactivity appearing in lipid thereafter. This was compatible with a single pool of phosphate within the tissue with a slow rate of turnover. When C^{14}-phosphatidyl-choline is administered in the diet, the dietary contribution to milk phospholipid was calculated to be approximately 0.05%. Likewise, the uptake of P^{32}-lipoprotein by mammary tissue does not result in labelled milk phospholipids. The synthesis of phospholipid by isolated cultured mammary cells occurs from glycerol (Kinsella, 1968).

4. Lactogenesis and the Secretion of Colostral Fat The foregoing discussion dealt mainly with the synthesis of mature milk, and little

consideration has been given to lactogenesis. In general, the enzymatic activity of mammary tissue increases at or near parturition and coincides with the appearance of milk. In cow mammary tissue, the rate of lipogenesis as measured by cytosol enzyme activities increases by 10- to 40-fold from 30 days pre-partum to 40 days post-partum (Mellenberger *et al*, 1973). Accompanying this is a change in the fatty acid pattern towards an increase in short and medium chain fatty acids, similar to the distribution typically found in milk fat (Glass *et al*, 1967). Rabbit mammary tissue displays similar behaviour, with the fatty acid pattern of mid-pregnancy milk being characteristic of cellular lipids rather than milk fat (Strong and Dils, 1972). Changes in the lipogenic enzyme activity of mammary tissue have also been demonstrated in rat (Baldwin and Milligan, 1966; Howanitz and Levy, 1965), mouse (Jones, 1972) and rabbit (Hartmann and Jones, 1970). Read and Sarriff (1965) considered that the change in fatty acid composition antepartum to mid-way through the first week post partum, with increasing levels of lauric and myristic acids, is evidence of increasing intramammary synthesis in human milk, most of the fatty acids of early colostrum being derived from plasma.

Lipoprotein-lipase activity increases in mammary tissue 2-3 days pre-partum and is maintained at a high level throughout lactation (McBride and Korn, 1963; Robinson, 1963; Otway and Robinson, 1968; Hamosh *et al*, 1970). Lipoprotein lipase activity in the gland depends to some extent on prolactin (Zinder *et al*, 1974).

E. CHOLESTEROL AND BILE ACID METABOLISM

1. Synthetic Pathways The synthesis of cholesterol in an animal system utilizing acetate as the substrate was first demonstrated by Bloch and Rittenberg in 1942 using deuterated-acetate. The multiple steps in the biosynthetic pathway have, in the main, been demonstrated subsequently, and have been reviewed by Holloway (1970). The pathway consists of the formation of Acetyl CoA from acetate, with Acetoacetyl CoA

proceeding to β-hydroxy-β-methyl glutaryl CoA (HMG-CoA) and the irreversible formation of mevalonic acid. The subsequent steps are not as open to physiological influences as those prior to mevalonate formation (Gould and Popják, 1957; Bucher *et al*, 1957; Siperstein and Guest, 1960), the early chemical reactions being common to other metabolic pathways (Bortz, 1973). Following mevalonate formation, phosphorylation and condensation results sequentially in squalene, lanosterol and cholesterol.

One of the functions of cholesterol is that of precursor for the production of steroid hormones. the recovery of deuterated-pregnandiol from the urine of a woman 8 months pregnant following administration of deuterated-cholesterol, prompted Bloch (1945) to suggest the *in vivo* synthesis of steroid hormone via cholesterol degradation. Demonstrations of the derivation of pregnenolone from cholesterol, the former being a precursor of the corticosteroids (Lynn *et al*, 1954), and the pathways of androgen and oestrogen synthesis have since occurred (Samuels and Eik-nes, 1968; Holloway, 1970). Under normal conditions, however, transformation of cholesterol into adrenal or gonadal hormones would probably account for less than 10% of the total daily cholesterol production (Miettinen, 1970).

Cholesterol is the precursor of a second group of sterols, namely the bile acids, the latter also serving as a major route for the excretion of cholesterol. The transformation of cholesterol into bile acid was first observed in the dog when injection of deuterium-labelled cholesterol gave rise to deutero-cholic acid (Bloch *et al*, 1943). The role of the liver in this degradation was confirmed by Harold *et al* (1955) using the isolated perfused rat liver. The liver is responsible for the production of the primary bile acids, (cholic acid and chenodeoxycholic acid in man), which arise via a stepwise pathway following 7α-hydroxylation of cholesterol (Danielsson and Tchen, 1968) and this is followed by their

conjugation with taurine and glycine (Harold *et al*, 1955). Secondary bile acids arise from the primary acids as the result of degradation by microorganisms in the caecum and large intestine. The conjugated primary bile salt is first hydrolyzed to release the free acid and then the 7α-hydroxyl group is removed from cholic or chenodeoxycholic acids to give rise to deoxycholic and lithocholic acids respectively. Some of the secondary bile acid is reabsorbed and reconjugated in the liver (Hayakawa, 1973). While cholesterol gives rise to the bile acids, bile acid regulates the absorption of cholesterol and the two sterols participate mutually in the control of their production.

2. Cholesterol Synthesis

The Site of Synthesis. All tissues have been found capable of cholesterol synthesis with the exception of the adult central nervous system (Srere *et al*, 1950; Dietschy and Siperstein, 1967). The most active sites are the liver and the small intestine, and together these are responsible for 80% and 97% of the total synthesis in rat and monkey respectively (Dietschy and Wilson, 1968), when the animals are maintained on low cholesterol diets. In the monkey, 82% was attributable to the liver.

Considering cholesterol synthesis at a cellular level, compartmentalization of the synthetic pathway exists. The initial condensation of acetyl CoA occurs within the cytosol, whilst the enzyme responsible for converting HMG-CoA to mevalonate, namely HMG-CoA reductase, is found almost exclusively in the microsomes (Bucher and McGarrahan, 1956; Siperstein and Fagan, 1966). This is ultimately where newly synthesized cholesterol is recovered (Bucher and McGarrahan, 1956) and where most cellular cholesterol is to be found (Rice *et al*, 1953).

Regulation of Synthesis. The regulation of cholesterol synthesis was deduced by Gould and Popják (1957) to occur early in the pathway, prior to mevalonate formation, and was subsequently demonstrated to occur at the level of the reduction of HMG-CoA to mevalonate (Siperstein and

Guest, 1960). Bucher *et al* (1960) showed HMG-CoA reductase activity to be decreased in the fasting animal, whilst more recently, Regen *et al* (1966) have shown that this was due to decreased enzyme synthesis. Steps further along the pathway of cholesterol synthesis may also be concerned with regulation (Gould and Swyryd, 1966).

Bile acids may inhibit cholesterogenesis (Beher and Baker, 1958; Grundy *et al*, 1966) at the level of HMG-CoA reductase (Back *et al*, 1969; Hamprecht *et al*, 1971), with an additional effect on bile acid synthesis itself, probably at the level of 7α-hydroxylase (Shefer *et al*, 1969). These studies are sometimes difficult to interpret, however, there being the additional change in cholesterol absorption. This objection is overcome in studies utilizing lymphatic diversion (Hamprecht *et al*, 1971) or ileal exclusion (Grundy *et al*, 1971), when it is clear that disruption of bile acid metabolism affects cholesterol synthesis directly, without altering cholesterol absorption.

Excretion of Cholesterol. Cholesterol is excreted via 2 main routes, the intestine and the skin. The skin loss is via degradation of superficial cells, and under most conditions probably occurs at a constant rate. That excreted through the intestine is in 2 forms, neutral sterols (comprising cholesterol and degradation products) and acidic sterols (the bile acids).

The cholesterol in faeces may be both endogenous and exogenous, the latter dietary cholesterol that had not been absorbed. Endogenous faecal cholesterol arises from biliary secretion and intestinal synthesis, the latter being mainly from shed mucosal cells. Just as bile acids are subject to bacterial degradation, so cholesterol may give rise to several products. In normal adult animals, the most common excretory product in the neutral sterol fraction is coprostanol, with cholesterol being present in fairly small quantities. In germ-free animals, or in those treated with antibiotics, coprostanol is entirely absent or decreased

substantially (Coleman and Baumann, 1957; Danielsson and Gustasson, 1959).

A third, and minor route, is that of the excretion of hormones, often via the kidney, following detoxification in the liver.

3. Bile Acid Synthesis

The Site of Synthesis. In isolated perfused rat liver preparation, taurochenodeoxycholic acid is the main end product, exceeding taurocholic acid (Harold *et al*, 1955). This is the converse of the situation in the intact animal. Bile acids formed with the liver are, by definition, primary acids, whilst secondary bile acids are those formed during enterohepatic circulation. Their formation within the intestinal lumen is due to bacterial action and several anaerobic microorganisms found as normal flora within rat and human intestine have been found to degrade primary acids, e.g. Bacteroides, Clostridium, Enterococcus, Lactobacillus (e.g. Midtvedt and Norman, 1967). The extent to which microbiological degradation occurs varies considerably between species, as well as between animals of the same species. Additional factors include changes in flora caused by diet, changes in bile acids caused by diet, and the rate of faecal flow (Bergström and Danielsson, 1968).

It should be mentioned at this stage that, in some species (rat and mouse) (Bergström *et al*, 1960), chenodeoxycholic acid may be further metabolized by the liver, and by definition, these products are also primary bile acids. In rat liver, a mitochondrial system producing lithocholic acid from cholesterol has been described by Mitropoulos and Myant (1966), this being usually considered a secondary bile acid.

Bile acid formation probably begins in the microsomes where the changes in the steroid nucleus, such as 7α-hydroxylation occur; oxidation of the side chain takes place in mitochondria (Bergström and Danielsson, 1968). The conjugation of bile acids by taurine and glycine involves activation of the carboxyl group, catalyzed probably by the microsomal fraction, with subsequent conjugation being associated with lysosomal

activity (Scherstén, 1967).

Regulation of Synthesis. The site of control of bile acid biosynthesis has been suggested as lying at the first steps in the pathway, that of 7α-hydroxylation. Early, Bergström and Danielsson (1958) showed that homeostatic control was dependent on the return of bile acids to the liver via the portal blood. Drainage of bile acids via bile fistulae is followed by a several-fold increase in bile acid production due to interruption of bile acid resorption. This has been shown in liver homogenates (Danielsson *et al*, 1967) and in the whole rat (Shefer *et al*, 1969). In the latter experiments, intraduodenal infusion of taurocholate resulted in depression of the accelerated bile acid production brought about by a biliary fistula. The same authors later presented (1970) further evidence that 7α-hydroxylase was capable of regulating bile acid biosynthesis via feedback control, but only if hepatic cholesterol synthesis was "adequate". They concluded that this involved enzyme synthesis and degradation as with HMG-CoA reductase (Regen *et al*, 1966).

The Enterohepatic Circulation and Excretion of Bile Acid. At least 95% of bile acids excreted in bile are resorbed in the gut. In animals, bile acid absorption has been demonstrated along the whole length of the small intestine, utilizing various transport mechanisms according to the site and the bile acid concerned (Dietschy, 1968). The situation seems to be substantially the same in the adult human. There is little net absorption of bile acid from the proximal small intestine, as demonstrated by perfusion studies (Borgström *et al*, 1963; Simmonds *et al*, 1967), a fact well supported by the marked increase in bile acid turnover seen in patients who have undergone ileectomy (Hofmann and Grundy, 1965; Hardison and Rosenberg, 1967). Both perfusion studies used taurine conjugates, whereas if the glycine compounds are used, passive absorption occurs in the jejunum (Hislop *et al*, 1967). Of that small percentage of bile acid which escapes into the caecum, most will undergo transformation into

secondary acids, with a relatively small amount of primary bile acid detectable in faeces (e.g. Danielsson *et al*, 1963). Colonic resorption of bile acid is well documented (Mekhjian *et al*, 1968), but is less efficient than that in the small intestine, due to the unfavorable physical state of some of the acids, such as lithocholic, which tends to precipitate in the colonic environment. After continuous biliary drainage for a few days, secondary bile acids tend to disappear from bile (Samuel *et al*, 1968). Resorbed bile acids and salts are carried back to the liver via the portal blood. Under normal circumstances only low concentrations can be detected in the peripheral circulation (Sandberg *et al*, 1965; Simmonds *et al*, 1973). Both conjugated and free acids are taken up by the liver, the latter being conjugated (if secondary) or reconjugated (if primary) prior to re-excretion into bile.

Using isotopic dilution techniques, it has been estimated that in the adult human the bile acid pool is 2-5 g (e.g. Lindstedt, 1957), and that circulation is of the order of 5 to 10 times per day (Hofmann, 1967), with the component bile acids having different half-lives (that of cholic acid being shorter than that of chenodeoxycholic acid).

F. THE ORIGIN OF FOETAL CHOLESTEROL BILE ACIDS AND BIOSYNTHESIS IN THE YOUNG

1. Cholesterol Theoretically the origin of foetal cholesterol is two-fold, from *de novo* synthesis within the foeto-placental unit and from the maternal circulation. This problem has been considered by many workers but generally in non-primates, though in the last 10 years both human and sub-human primate studies have been carried out.

A limited degree of transfer in the rat, amounting to about 11% of total foetal cholesterol, was demonstrated using deuterium-labelled cholesterol injected into the mother (Goldwater and Stetten, 1947). Chevallier (1964) using cholesterol-4-^{14}C, showed a diminishing contribution from the maternal circulation as the foetus matured: whereas at term

approximately 15-20% was derived from the mother, as much as 60-70% was of maternal origin in the 12 day foetus (about mid-gestation). These results contrast with those of Popják and Beeckmans (1950), who were unable to demonstrate transfer of cholesterol from the rabbit's maternal circulation to the foetus. Having shown that the foetal liver was capable of synthesizing cholesterol from acetate, they concluded that all foetal cholesterol was derived from within the foetus. However, since the foetal rabbit's plasma cholesterol exceeded that of the mother, the higher specific activity within the foetus did not exclude synthesis in the mother and some transfer to the foetus. This view is supported by Connor and Lin (1967), who showed substantial transfer of maternal cholesterol across the placenta in both rabbit and guinea-pig. *In vitro* synthesis of cholesterol in foetal liver and placenta of sheep and pig has also been demonstrated (Nitchuk and Ainsworth, 1972). Foetal cholesterol is probably derived from endogenous synthesis and from the mother's circulation.

Studies in the human have been carried out by a group of workers in Scandinavia. They have utilized a technique of perfusing either the foetus, the placenta or the combined foeto-placental unit, the specimens being obtained at hysterotomy during therapeutic abortion. In 1967, Solomon *et al*, using mid-trimester foetuses at 18-22 weeks of gestation, showed that both the foetal adrenal and liver were capable of cholesterol synthesis from acetate. This has been confirmed several times using similar preparations (Mathur *et al*, 1970; Telegdy *et al*, 1970a). The foetal liver and adrenal account for 98% of the synthesis under these conditions (Telegdy *et al*, 1970a) and acquire the ability to synthesize cholesterol very early in their uterine existence. Liver and adrenal tissue from foetuses of 11 to 14 weeks' gestational age incorporated large amounts of acetate into cholesterol. The rate of synthesis within the liver was some 5-9 times that in the adrenals per gram of tissue (Givner and Jaffe, 1971). These organs are also the most active sites

for *de novo* synthesis of cholesteryl esters in the mid-gestation foetus (Ćekan *et al*, 1973), the main area in the adrenal being the foetal zone (Isherwood and Oaken, 1972).

The role of the placenta as an endocrine organ has become increasingly recognized, for instance its synthesis of pregnenolone from cholesterol (Telegdy *et al*, 1970b). It is of interest, therefore, to define its capacity for *de novo* synthesis of cholesterol as a source for this role as well as for the growing foetus. Cholesterol synthesis may be governed by the age of the placenta; for instance at term, the placenta is able to synthesize small amounts of cholesterol from acetate (van Leusden and Villee, 1965; Zelewski and Villee, 1966; Boguslawski and Zelewski, 1971) and from mevalonate (Zelewski and Villee, 1966), whilst perfusion experiments of either the mid-trimester placenta or foeto-placental unit have been unable to demonstrate significant *de novo* cholesterol production (Telegdy *et al*, 1970a; van Leusden *et al*, 1971).

The relative importance of foetal synthesis of cholesterol and transfer from the maternal circulation has been studied in non-human primates, which may only give an indication of the human situation (taking into account wide species differences) and in a small group of human subjects. In the latter, either a therapeutic abortion was to be carried out or the subject was known to be carrying a foetus that would not live after birth, for instance, an anencephalic.

Pitkin *et al* (1972) using the rhesus monkey showed that maternal transfer of cholesterol accounted for some 42.6% of serum cholesterol in the foetus at term. There was a strong net flux of cholesterol across the placenta from the maternal to the foetal circulation, labelled cholesterol being detected on the foetal side less than 24 hours after administration to the mother. Equilibration of the two specific activities occurred after 10-12 days, whilst maternal serum cholesterol attained a specific activity only 5% of the foetus if the latter were labelled *in*

utero. The difference in total plasma pool between mother and foetus could not account for all this difference. Plasma cholesterol concentration in the mother was 80.3 ± 18.5 mg/100 ml and in the foetus 59.6 ± 15.6 mg/100 ml. The baboon has also been used in placental transfer studies. Khamsi *et al* (1972), using 2 animals, showed the foetal esterified plasma cholesterol to have a lower specific activity than non-esterified cholesterol. The liver was the major site of foetal cholesterol synthesis.

The anencephalic foetus at term has been used to study the human situation *in utero*. Hellig *et al* (1970) published a report of 2 such cases, administering labelled cholesterol to the mother during the forty-first week of gestation, 8 and 3 days prior to delivery. At the time of delivery, the plasma specific activity of the first foetus was 18% that of the maternal level; the second was very much lower. The esterified cholesterol isolated from foetal plasma and liver and placenta showed only half the specific activity of free cholesterol. These figures are in good agreement with those of Davis *et al* (1956) and Plotz *et al* (1968). This group demonstrated that some 20% of foetal cholesterol arises from the maternal circulation in the mid-trimester.

Negative feedback control of synthesis seems to operate in the foetus in view of the findings of Connor and Lin (1967), that an increased proportion of foetal cholesterol is derived from the maternal circulation if the mothers had been fed cholesterol-containing diets during pregnancy. But do changes in cholesterol synthesis contribute to the rise in plasma cholesterol concentration after birth? Carroll (1964) compared the incorporation of C^{14}-acetate into cholesterol *in vitro* and *in vivo* in the liver of suckling and weanling rats. Incorporation was significantly lower in the suckling animals. However, if mevalonate was used as the precursor, *in vitro* the 2 groups of animals gave substantially the same incorporation into cholesterol. The control point seemed to lie therefore

prior to the production of mevalonate. If the older suckling animals had access to the adult diet, there was little difference in the utilization of acetate. Ballard and Hanson (1967), considering that glucose was a more physiological substrate, compared it and acetate (both labelled) in the foetal (18 days' gestation), neonatal, suckling and adult rat. Their results generally showed that the incorporation of both precursors was greatest in the foetus, decreasing sharply in the neonate and with suckling, but then increasing slightly upon weaning to reach adult values.

Further evidence for the control point lying prior to mevalonate synthesis was furnished by Harris *et al* (1967). McNamara and his colleagues (1972) have studied the developmental patterns of hepatic HMG-CoA reductase in the rat. Because of the known diurnal variation of HMG-CoA reductase activity in the adult, a standard time of 12 noon was adopted for assay. A sharp rise in activity was seen in the foetus just prior to term, but this was short-lived, and followed by a rapid decline in the post-natal period. For the first 8 days of the rat's extra-uterine existence, activity was maintained at adult levels, and then declined further, to a very low level until weaning. An overshoot in activity occurred on weaning, of about 2 weeks duration and it could be advanced or retarded by changing the time of weaning. In the same paper, the authors explore changes in the diurnal variation of HMG-CoA reductase activity. In the 6 day old suckling rat, there is an inversion of the rhythm, with peak activity occurring in the 12 noon-4 p.m. period, which probably reflects the suckling's feeding pattern, most milk being drunk during daylight hours, in contrast to the adult nocturnal habit (Greengard, 1970).

It has been suggested that in the pathway of cholesterol synthesis in the adult liver, control points exist beyond HMG-CoA reductase, and with this in mind, work has been carried out in the young animal. Shah

(1973), using mevalonate as precursor, has demonstrated low incorporation *in vitro* into both non-saponifiable and digitonin-precipitable lipids in the suckling rat compared with the weaned rat. The author considered that the cholesterol content of milk acted to inhibit endogenous cholesterol synthesis. Wróbel (1972), examining 7-dehydrocholesterol reductase as a possible rate-limiting enzyme in the perinatal animal reported that activities of this enzyme were low in the foetal and newborn rat, and increased sharply to adult levels between days 19 and 22. However, since 7-dehydrocholesterol does not accumulate in the newborn rat liver the increase in activity of 7-dehydrocholesterol reductase coinciding with weaning and maturation of other hepatic enzymes of lipogenesis (Ballard and Hanson, 1967) probably reflects an overall increase in cholesterogenesis.

The question that remains is the nature of the inhibitor that reduces HMG-CoA reductase activity during suckling. Incubation with either a supernatant fraction of the suckling liver homogenate or pre-incubation with rat milk decreased the activity of adult liver HMG-CoA reductase (McNamara *et al*, 1972). The inhibitory activity of the milk was non-dialyzable, relatively heat stable and was associated with the protein fraction. A recent paper by Boguslawski and Wróbel (1974) presents evidence for the existence of a similar inhibitor in cow's milk and human milk. In their experiments, they used 5-6 week old male rats and studied the inhibition *in vivo* and *in vitro*. *In vitro*, the production of digitonin-precipitable sterols from acetate and mevalonate was decreased by the addition of small amounts of cow's or human milk, fresh or boiled. Further examination of cow's milk showed that the inhibitor was probably not associated with protein, remaining in the supernatant after 5% trichloracetic acid or lactic acid precipitation. *In vivo*, when mature animals were fed on cow's milk or a diet supplemented with cow's milk, the livers showed a 50% decrease in acetate, but not mevalonate,

incorporation into sterols.

Some other inhibitors of sterol synthesis, *in vivo* and/or *in vitro* which have been tentatively identified, and which may operate in the neonatal period, are the bile acids (e.g. Harris *et al*, 1967) and cholesterol itself (Shah, 1973). These are included in Table 1.12.

In summary then, the biosynthetic pathway for cholesterol synthesis is essentially the same as in the adult liver. Marked changes in enzyme activity occur during the transition from foetus to neonate and from suckling to juvenile animal. Control occurs at the level of HMG-CoA reductase as well as beyond, though the later rate-controlling enzymes are not clearly defined. Slow bile acid turnover in the suckling rat may be involved in negative feedback control (Harris *et al*, 1967), as may cholesterol itself, and other milk constituents yet to be fully characterized and evaluated.

The Role of Diet and Intestinal Micro-Organisms. Another major dietary change at the time of weaning is the increase in bulk, including fibre. The importance of the fibre content of diet in modifying experimental atherosclerosis has been demonstrated in rabbits (Kritchevsky, 1964; Kritchevsky and Tepper, 1965, 1968; Moore, 1967). It may be due to the binding of bile salts to non-nutritive fibre (Kritchevsky and Story, 1974). The excretion of sterols and bile acids is higher in animals fed on chow-type diets (Portman and Murphy, 1958; Bloomfield, 1963; Horlick *et al*, 1967), and the low excretion of bile acid in the suckling rat (Harris *et al*, 1967) may be related to the absence of fibre. The increased fibre-bound loss of sterols at weaning may stimulate synthesis of cholesterol and bile acids, while at the same time lower the plasma cholesterol.

It is difficult to separate the effects of changes in dietary composition and changes in bacterial flora of the gut, since a change in diet often alters the latter (e.g. Crowther *et al*, 1973). The infant's

TABLE 1.12

Possible Influences Upon Sterol Synthesis in the Young Animal

Source of Material	Characteristics: Dialyzable	Precipitable	Heat Resistant	Known or Possible Action
NEONATE - *in vitro*				
Suckling rat liver-supernatant from homogenate	-	+[$(NH_4)_2SO_4$]	+	↓ Acetate conversion to mevalonate[1]; ↓ HMG CoA reductase activity[2]
Rat milk	-	+[ethylene/ether]	+	↓ HMG CoA reductase activity[1]
Cow's milk	+	-[TCA]	+	↓ Acetate and mevalonate conversion to digitonin-precipitable sterols[3]
Human milk			+	↓ Acetate and mevalonate conversion to digitonin-precipitable sterols[3]
NEONATE - *in vivo*				
Rat milk in suckling animal				↓ HMG CoA reductase activity[2]
Cow's milk in 5-6 week old rat				↓ Acetate conversion to digitonin-precipitable sterols[3]
Bile - including cholic acid				↓ Acetate conversion to mevalonate[1]

Continued

TABLE 1.12

(Continued)

Source of Material	Characteristics: Dialyzable	Characteristics: Precipitable	Characteristics: Heat Resistant	Known or Possible Action
ADULT - *in vitro*				
Bile acids				↓ Acetate conversion to mevalonate[1]
Bile protein				↓ Acetate conversion to cholesterol[4]
ADULT - *in vivo*				
Dietary cholesterol (short-term)				↓ HMG CoA reductase production[5]
Bile acids				↓ Acetate conversion to mevalonate[1]

[1]Harris *et al* (1967)

[2]McNamara *et al* (1972)

[3]Boguslawski and Wróbel (1974)

[4]Ogilvie and Kaplan (1966)

[5]Shapiro *et al* (1971)

gut at birth is sterile, but within hours bacterial colonization has occurred (Davies, 1971). The total faecal bacterial count in a human baby is maximal by day 2 post partum, and does not change significantly over the next year (Smith and Crabb, 1961). The individual composition does vary however, with, for instance, a decrease in E. coli. In the adult, cholesterol undergoes microbiological degradation in the gut, most commonly to coprostanol. Analysis of faecal sterols in the young human infant shows an absence of coprostanol. It appears in some babies at about 5 months, and is usually present in reasonable quantities by the time the infant is one year of age, the percent conversion of cholesterol to coprostanol varying from 0 to 83% in infants 52-90 weeks of age (Gustafsson and Werner, 1968; Gustafsson and Sjövall, 1969; Gustafsson *et al*, 1970). This makes an interesting contrast to the findings in the weanling rat which does excrete coprostanol (Coleman and Baumann, 1957).

2. Bile Acids Bile acid metabolism in the neonate and young child has received most attention in terms of its role in fat absorption, particularly since lipid absorption is relatively inefficient in the very young.

Information regarding the component bile acids of the newborn has been obtained from post-mortem studies, duodenal intubation and faecal and plasma analysis. Poley *et al* (1964) found that the principal bile acids of the mid-trimester foetus, 22 to 26 weeks' gestation, were dihydroxy acids, conjugated with taurine, whilst a slightly older foetus (28 weeks) showed a predominance of taurocholic acid with very small amounts of glycochenodeoxycholic acid. This predominance of taurine as the conjugating amino acid of bile acids is the reverse of that found in the adult, in whom the ratio of glycine to taurine conjugates is of the order of 3:1.

In the neonate both primary and secondary bile acids are present during the first week of life, as demonstrated by studies of the meconium (Sharp *et al*, 1971; Back and Ross, 1973), duodenal contents (Poley *et al*, 1964; Bongiovanni, 1965) and serum (Sandberg, 1970). The major primary acid of the first week of life is cholic acid, the ratio of cholic to chenodeoxycholic acid being 2.5:1 (Encrantz and Sjövall, 1957; Watkins *et al*, 1973a). Over the next month, with increasing maturity, the ratio decreases, becoming 1.2:1 (adult proportion). Only very small amounts of deoxycholic acid, or none at all, have been detected in neonates (Encrantz and Sjövall, 1957, 1959).

Synthesis of bile acids in the foetal liver may occur via at least 2 pathways. The first is the accepted adult sequence, resulting in cholic and chenodeoxycholic acids. The second pathway utilizes a system demonstrated in the rat by Mitropoulos and Myant (1967), in which lithocholic acid and 3β-hydroxy-5-cholenoic acid occur as intermediates, and which may be a minor pathway in the adult liver (Anderson *et al*, 1972). Whilst 3β-hydroxy-5-cholenoic acid has been detected in the plasma of women with recurrent cholestasis of pregnancy (Back *et al*, 1972), it could not be found in normal subjects. It is present in higher amounts in the meconium of the premature infant as compared with the mature neonate (Back and Ross, 1973). Further evidence for the pathway in the immature human liver is provided by the finding of large amounts of 3β-hydroxy-5-cholenoic acid in the urine of infants suffering from liver disease, including extrahepatic biliary atresia (Makino *et al*, 1971). This seems to make placental transfer from the maternal circulation of this intermediate unlikely. However, the latter mechanism must be considered to account, at least partly, for the presence of other minor constituents, such as deoxycholic acid (Lester *et al*, 1972).

The Enterohepatic Circulation and the Bile Acid Pool. Maintenance of the bile acid pool requires a delicate balance between rates of synthesis

and excretion, and the coordination of several organs and enzyme systems. In the adult this is achieved by the enterohepatic circulation of bile acids. Many functions of the foetal and neonatal liver are extremely immature, and maturation of the enzyme systems may not occur until post-natally.

There is little direct information on the enterohepatic circulation in the human infant. Some earlier published studies of duodenal bile indicated that bile acid concentrations in samples obtained without stimulation of the gallbladder were similar to those in adults or older children (Poley *et al*, 1964; Encrantz and Sjövall, 1959). However, bile acid concentrations within the gallbladder are lower than in the adult (Encrantz and Sjövall, 1957), and recent work suggests that the intraduodenal concentration is relatively low (Lavy *et al*, 1971), in premature infants being less than the critical micellar concentration after a meal (Signer *et al*, 1974).

The foetal dog has been used as a model for the investigation of bile acid metabolism in a series of experiments by Lester, Smallwood and colleagues. The liver of the foetal dog, when examined near term, is able to take up, conjugate and secrete tracer doses of cholic acid (Jackson *et al*, 1971). The existence of a foetal enterohepatic circulation was demonstrated by the administration of a taurocholate load, which was excreted by the liver and concentrated within the gallbladder, then resorbed (Smallwood *et al*, 1972). Absorption of bile acids is not fully developed within the ileum of the foetus, and the jejunum absorbs nearly as much as the ileum (Smallwood *et al*, 1970). Conversion of labelled cholesterol into bile acids enabled estimates of the rates of synthesis and pool sizes of bile acids to be made (Lester *et al*, 1972). In studies lasting 36-53 hours, cholic acid was observed to be the major bile acid, with smaller amounts of chenodeoxycholic acid. Whilst the foetal pool size was reduced compared to the adult (on a weight basis), there was a

marked increase in synthesis of bile acid from cholesterol when the pool was depleted by biliary drainage. Placental transfer from the maternal circulation of cholic and deoxycholic acids was also shown. Deoxycholic acid was not synthesized in the foetal liver.

Understandable reluctance by investigators to use radioactive isotopes in infants and children has limited data on bile acid kinetics. Watkins *et al* (1973a) have recently used deuterated-bile salts to overcome this problem. Five full-term infants were studied between 2 and 8 days of age, with samples either by total faecal collection (3 infants) or duodenal sampling over 5 consecutive days (2 infants). A mean value of 41.4 mg was found for the size of the cholate pool, with a rate of synthesis of 22.7 mg/day. No measurement of chenodeoxycholate kinetics was made, apart from the relative amounts of the 2 primary bile acids in faeces. The data was expressed in terms of body surface area, which is proportional to the metabolic rate of an organism (Kleiber, 1947) and provides better comparison between adults and children. The cholate pool was 290 mg/m^2 and the synthetic rate 110 mg/m^2/day, both values being about half of those in adults. In further studies, Watkins *et al* (1973b) have extended their technique to 4 premature infants of 34-36 weeks' gestation. Synthesis of cholate was found to be 6.3 mg/day and that of chenodeoxycholate 1.6 mg/day; the pool sizes were 15 and 3.8 mg respectively, that is half the value for the full-term infants.

In the adult, in the steady state, the rate of bile acid excretion is equal to the rate of synthesis. In a young infant, whose rate of growth precludes him being truly in a steady state, the measurement may only give an indication of the true value. Few values of faecal bile acids are available. Weber *et al* (1972, 1973) in 18 normal infants and children, varying in age from 4 months to 5 years (mean age 2.4 years) obtained values of 18.3 mg/kg body weight/72 hours (or 110 mg/m^2/day). This is identical to the rate of synthesis for cholate obtained by Watkins *et al*

(1973a). Leyland (1970) found mean daily excretion of faecal bile acid to be between 10 and 85 mg/day. The 16 children whom he studied were aged between 2 months and 14 years, and mean daily excretion "appeared to correlate with body weight".

It would appear then, that in the infant, bile acid synthesis is increased relative to the total pool size, in comparison with the adult, but that the rate is not sufficient to achieve optimal intraduodenal concentrations for fat absorption. Synthesis is probably under the same controls as in the adult, as demonstrated by the increased synthesis in foetal dogs during biliary drainage. Signer *et al* (1974) have demonstrated an increased loss of bile acid in premature infants fed a cow's milk substitute as compared with those on human milk. Sequestration of bile acid may occur in the unabsorbed lipid, particularly in the long chain fatty acids, which are poorly tolerated by the neonate (Fomon *et al*, 1970; Hann *et al*, 1970) and excreted as calcium soaps and glycerides (Watkins *et al*, 1974). A similar sequestration of bile acid is seen in children suffering from cystic fibrosis, when bile acid loss is increased several fold (Weber *et al*, 1973).

The bile acid pool in the neonate appears to have the same physiological confines as in the adult, as shown by measurements of bile acids in the peripheral circulation which are comparable to those of adults (Sandberg, 1970), and excretion of only trace amounts by the kidney (Norman and Strandvik, 1973).

The presence of secondary bile acids in the neonate has been attributed to placental transfer from the maternal circulation in the case of deoxycholic acid, and to possible foetal production of lithocholic acid. The level of deoxycholic acid decreases and then disappears from the plasma in the first few days of life (Sandberg, 1970) and then reappears in bile and faeces during the course of the first year (Encrantz and Sjövall, 1959; Poley *et al*, 1964; Sandberg, 1970). Recently Watkins

et al (1973b) detected small amounts of secondary bile salt in faeces at 7 days of age. Colonization of the gut by micro-organisms capable of 7α-hydroxylation of cholesterol is thought to coincide with the appearance of secondary bile acids (Eyssen, 1973). Overgrowth of bacterial flora, resulting in increased deconjugation of bile acids and formation of free bile acid may lead to bile acid loss in infancy (Watkins *et al*, 1973c).

G. THE EFFECT OF SEX STEROIDS ON CHOLESTEROL AND BILE ACID METABOLISM

Interest in this field is based on the finding that women of child-bearing age develop gallbladder disease more often than men of the same age (Kaye and Kern, 1971). Recently the results of clinical surveys have been released, the first concerning the incidence of gallbladder disease amongst users of oral contraceptives, and the second amongst those undergoing post-menopausal treatment with oestrogens (Boston Collaborative Drug Surveillance Program, 1973, 1974). In both there was a significant increase in the incidence of gallbladder disease (cholelithiasis and cholecystitis) amongst users as compared with age-matched controls [in the case of the oral contraceptive, 2-fold (1973) and oestrogens, 2.5-fold (1974)]. Among the users of oral contraceptives, those women aged 20-35 years and taking the "pill" for 6-12 months had a higher incidence than those who had taken it for a longer period of time. This has been attributed to the possibility that those destined to develop gallbladder disease will do so early in the course of therapy.

The apparent association of gallbladder disease with pregnancy led Riegel *et al* (1935) and Potter (1936) to analyze gallbladder bile obtained at the time of Caesarian section. Riegel *et al* (1935) reported an increase in cholesterol concentration coupled with a decrease in bile acid in 34 subjects, whilst Potter (1936) considered the bile composition to be abnormal, with the gallbladder generally large and distended. Large *et al* (1960), however, in repeating this experiment could detect

no statistical difference in cholesterol, cholate or phospholipid concentrations of gallbladder bile obtained at Caesarian section when compared with normal non-pregnant controls.

Comparing parameters of cholesterol and bile acid metabolism in men and women has shown some differences. There are altered proportions of cholesterol and bile acid present in the bile of some women (Grundy *et al*, 1972). A difference in the bile acids may also exist. Using needle aspiration of the gallbladder during abdominal surgery, bile samples were obtained from 12 females and 17 males, aged 20-72 years (Fisher and Yousef, 1973). The bile acid concentration was almost twice as great in the female patients (being 159 μ mole/ml compared to 86 μ mole/ml in the men). The contributions of cholic and chenodeoxycholic acid were 37% and 45% respectively in the women, being the reverse of that in the men, and having different proportions of taurine and glycine conjugates. Differences in the composition of rat bile attributable to sex have also been demonstrated (Fisher *et al*, 1972). In studies of sterol balance, when the difference in weight between men and women is taken into account, the apparent sex difference in the excretion of neutral sterols and bile acids is reduced (Grundy *et al*, 1972; Miettinen, 1973).

These studies and associations suggest a causal role for the sex steroids. Davis and Freston (1972) sampled duodenal bile in women aged 20-37 years, comparing a group taking oral contraceptives with one sampled on days 15-30 of a normal menstrual cycle. The groups were matched for age and gravity. They found that the cholesterol and bile acid concentrations were significantly elevated in the treatment group ($p < 0.005$), the cholesterol difference being more pronounced among young subjects. In a smaller group of women (3), hepatic bile was sampled from a tube left indwelling in the cystic duct following cholecystectomy (Pertsemlidis *et al*, 1973a). Using labelled cholic acid, the authors demonstrated an increase in pool size, synthesis rate and concentration

of cholic acid during treatment with mixed contraceptive steroid preparations.

The effect of ethinyl oestradiol on bile acid excretion in the rat has been explored by Davis and colleagues (1973, 1974). Bile acid synthesis was increased 4-fold in male Sprague-Dawley rats treated with ethinyl oestradiol, 0.5 mg/kg/day, whilst bile flow decreased to one-quarter. In a second series of experiments, treatment with 5 mg/kg/day produced a decrease in the degradation of cholesterol (19.8 ± 11.1 μ mole/day versus 41.7 ± 14.0). Concurrently the biliary cholesterol concentration increased 2-fold, whilst bile acid decreased by approximately 50%. Men given ethinyl oestradiol showed a marked increase in cholesterol turnover (Nestel *et al*, 1965).

There is little information available regarding the status of cholesterol and bile acid metabolism during pregnancy. Sjövall and Sjövall (1966) found that the concentration of bile acids in plasma was the same in pregnant and non-pregnant women, having a mean of 0.87 μg/ml (range 0.24-1.65) compared with normals of 0.82 μg/ml (Sandberg *et al*, 1965). There was no correlation between plasma levels and the time of gestation, and the concentrations of deoxycholic, chenodeoxycholic and cholic acids were approximately equal. In a single study during pregnancy, bile was sampled from an indwelling T-tube left *in situ* after cholecystectomy (Large *et al*, 1960). Biliary bile acid and cholesterol concentrations decreased in the second half of pregnancy, and increased in the first week post partum.

The most recent study which may be applicable to the human situation is one conducted in female baboons before and during pregnancy (Dietrick *et al*, 1973). Percutaneous gallbladder aspiration was utilized to investigate bile acid kinetics. During pregnancy, bile salt pool size and synthesis were reduced and half-life increased. A note of caution may be necessary, however, for the baboon undergoes a lowering of plasma

cholesterol during pregnancy in contradistinction to the human (van Zyl, 1957).

CHAPTER 2

THE HYPERLIPIDAEMIA OF PREGNANCY

INTRODUCTION

Historically there was early recognition of the hyperlipaemia of pregnancy, with John Hunter in the eighteenth century ascribing it to the absorption of chyle. Virchow in the nineteenth century demonstrated that it was due to lipid in the blood by clearing the plasma by the simple means of using the lipid solvent, ether. The theories as to its causation have been multiple (Boyd, 1934).

There is general agreement that in Western populations normal pregnancy leads to an increase in the major plasma lipids, which is followed by a decrease in the puerperium. There is some dispute as to the timing of the rises of individual plasma lipids. Plasma cholesterol may decrease during the first trimester of pregnancy, though still remain within the normal range for the population (de Alvarez *et al*, 1959). Subsequently the level increases, usually progressively throughout pregnancy, then either decreasing slightly prior to delivery (Oliver and Boyd, 1955) or remaining elevated during the whole of the last trimester (de Alvarez *et al*, 1959). The increase is of the order of 25%. Post partum there is usually a slow steady decrease (Watson, 1957) with blood lipids being still above normal several months after delivery (Peters *et al*, 1951; Oliver and Boyd, 1955; de Alvarez *et al*, 1959).

Most authors report a 2-3 fold increase in plasma triglyceride concentration during pregnancy (e.g. Cramér *et al*, 1964/5; Svanborg and Vikrot, 1965a; Knopp *et al*, 1973). The level decreases rapidly following delivery, being some 50% of the maximal value by day 7 post partum (Konttinen *et al*, 1964; Svanborg and Vikrot, 1965b).

The increasing density of the β band on lipoprotein electrophoresis with pregnancy (Watson, 1957) was confirmed by ultracentrifugation (Pantelakis *et al*, 1964a,b). The ratio of β/α bands was reported as being uniformly increased (e.g. Russ *et al*, 1954; Oliver and Boyd, 1955). A recent study by Knopp *et al* (1973) showed the triglyceride content of all

3 ultracentrifugal fractions to be increased (i.e. VLDL, LDL and HDL), whilst they could detect increases in cholesterol only in VLDL and LDL in the third trimester.

The effect of intervening factors on the normal pattern of changes has received some attention. In toxaemia of pregnancy plasma cholesterol and phospholipid levels have risen higher than in normal pregnancy in some studies, though the increase was not always significant (Boyd, 1934b; de Alvarez and Bratvold, 1961; Konttinen *et al*, 1964). Plasma triglycerides also tend to be higher (Nelson *et al*, 1966), whilst the placental triglyceride content is significantly higher (136 mg/100 g tissue in toxaemic pregnancy versus 89 mg/100 g in normal placenta).

Hormonal Effects. It is believed that the considerable hormonal changes that occur during pregnancy must influence lipid metabolism. The widespread use of steroid sex hormones, both post-menopausally and in the form of oral contraceptives, has given further opportunity for the study of hormonal effects. Whilst the levels of circulating hormone are different in pregnancy and on taking the oral contraceptive, the trend may be analogous.

There is a difference in the level of plasma lipids between males and females during the reproductive years (20-45 years) (Jones *et al*, 1951; Adlersberg *et al*, 1956; Lawry *et al*, 1957; Lewis *et al*, 1957), which disappears after natural (Schaefer, 1964; Carlson and Lindstedt, 1968) or surgical menopause (Oliver and Boyd, 1959; Aitken *et al*, 1971). Castration in the male also produces changes that can be predicted from the imbalance between oestrogen and androgen, β-lipoprotein being lower and α-lipoprotein higher than in age-matched normal males (Furman *et al*, 1957, 1958).

Administration of oestrogen to all groups, male and female, pre- and post-menopausal, normo- and hyper-cholesterolaemic, results in a decrease in plasma cholesterol (e.g. Eilert, 1953; Oliver and Boyd, 1956

a,b; Robinson and Le Beau, 1965; Aitken *et al*, 1971) and an increase in plasma triglyceride levels (Sznajderman and Oliver, 1963; Robinson and Le Beau, 1965; Aitken *et al*, 1971), which is large in the case of a pre-existant elevation (Zorilla *et al*, 1968). The lipoprotein pattern shows a decrease in LDL and an increase in HDL (Oliver and Boyd, 1954, 1956a,b; Hood, 1959; Robinson and Le Beau, 1965).

The administration of combined oestrogen-androgen preparations, as with the oral contraceptives, leads to an invariable increase in triglyceride levels, but inconstant changes in cholesterol. The more "oestrogenic" the preparation, the greater the triglyceride raising effect (Stokes and Wynn, 1971). The lipoprotein changes are governed by the relative proportions of the two hormones. The "oestrogenic" component raises VLDL (Wynn *et al*, 1966). Changes in HDL are variable; the more "androgenic" the activity, the lower the HDL concentration and the higher the LDL and total plasma cholesterol level (e.g. Aurell *et al*, 1966; Rössner *et al*, 1971). These compounds are orally active preparations such as methyl testosterone and 19-nortestosterone which increase LDL and decrease HDL (Oliver and Boyd, 1956a; Hood, 1959; Cramér, 1961, 1962); the naturally occurring androgens, testosterone and Δ-4-androstenedione, cannot reverse an oestrogen change (Hood, 1959).

Triglyceride transport has been studied by isotope kinetics. Turnover is profoundly influenced by gonadal hormones, the clearance from plasma being greater in women than in men in both normo- and hyper-triglyceridaemic subjects (Nikkilä and Kekki, 1971; Olefsky *et al*, 1974). Use of combined oral contraceptives doubles triglyceride production (Kekki and Nikkilä, 1971a) due to the action of the oestrogenic component (Kissebah *et al*, 1973). Clearance of endogenous and exogenous triglyceride is enhanced by the progestational drugs (Kikki and Nikkilä, 1971a; Kissebah *et al*, 1973) and by oxandrolone, an anabolic androgen (Glueck *et al*, 1973b).

This chapter describes the changes in concentration and distribution of cholesterol and triglyceride between the lipoprotein fractions of plasma during pregnancy and in the puerperium. The effect of the occurrence of pre-eclampsia as a complication in late pregnancy upon this pattern has been studied. Pre-existent hypercholesterolaemia in women will also profoundly influence the observed changes. The control group is of the effect of the hormonal change of the combined oral contraceptive upon the plasma lipids in young women.

METHODS

A. EXPERIMENTAL DESIGN

1. Plasma Lipid and Lipoprotein Concentrations During Pregnancy and in the Puerperium

Serial Changes During Pregnancy. The subjects for this study were derived mainly from one private obstetric practice. The group consisted of both nulliparous and multiparous women, the only criterion for inclusion in the longitudional study being their cooperation with regard to repetitive testing during pregnancy and in the puerperium. A total of 43 women were studied, their ages ranging from 20 to 41 years (mean 27.2 years). They were studied in a free-living situation, with routine ante-natal care. Apart from control of excess weight gain, no dietary instruction was given.

Blood samples were collected after an overnight fast, at 4-6 week intervals. Samples were obtained for the first time from several women on admission to hospital for induction of labour, or in labour. This group included women with pre-eclampsia, several of whom were referred from other practitioners. These latter samples were not necessarily in the fasting state. However, as may be seen in the results section, there are no statistical differences between the fasting lipid levels close to term and those at induction of labour, early in labour, or at delivery.

Associated with delivery, blood samples were taken

(1) at the time of delivery,

(2) the following morning following an overnight fast, within 12-24 hours of delivery (designated day 1), and

(3) on the fifth day post partum.

Following discharge from hospital further samples were taken 6-7 weeks post partum, and at 12 months.

The blood samples were immediately placed on ice, and kept at 4°C until lipid extraction was completed. The plasma lipoproteins were separated by a combination of preparative ultracentrifugation and precipitation as will be described below.

Factors That May Influence Lipid Metabolism During Pregnancy. The plasma concentrations of cholesterol and triglyceride at delivery and in the puerperium were measured in a second larger group of women (n=86). Record was made of the parity, labour, any coexisting conditions such as pre-eclampsia and age. Retrospectively an attempt was made to define maternal factors which may influence the concentration of plasma lipids at delivery.

2. Changes in Plasma Lipids Associated with the Use of a "Low Dose" Oestrogen Oral Contraceptive To complement the studies during pregnancy, the plasma lipoproteins from a small group of women were analyzed before and after commencing use of a mixed oral contraceptive (Nordiol 28, Wyeth Pharmaceuticals Ltd.). The girls were attending the University Student Health Service and had not used oral contraceptives recently. Their ages ranged from 17-22 years. The week prior to their next expected period and immediately prior to commencing taking the oral contraceptive, a blood sample was taken following an overnight fast. A second sample was taken during the third week of the first cycle on the "pill".

B. LABORATORY TECHNIQUES

1. Plasma Cholesterol and Triglyceride Concentrations These were measured in a Technicon Auto Analyser II (Manual, 1971). This utilizes the Lieberman-Burchard reaction for colorimetric estimation of cholesterol. Triglycerides are determined by fluorimetry, using a modification of the method of Kessler and Lederer (1965).

Known standards of both cholesterol (SMA Reference Serum, Extract cholesterol) and triglyceride (Triolein, Calbiochem) were treated in the same way as test samples. An 0.5 ml aliquot of plasma was extracted in 9.5 ml of isopropanol (redistilled — all isopropanol required redistillation to decrease high background fluorescence). Following addition of 2 gm of zeolite mixture (zeolite mixture — zeolite:Lloyd reagent:Cupric sulphate:Calcium hydroxide; 400:20:10:20 (w:w)), the samples were rotated for 30 minutes, then spun at 4°C at 2,000 rpm for 20 minutes (MSE, Mistral 6L centrifuge). The supernatant was decanted off.

2. Separation of Plasma Lipoproteins Plasma lipoproteins were separated according to their flotation into very low density lipoprotein (VLDL), having a density greater than 1.006, low density lipoprotein (LDL) of density 1.006-1.063, and high density lipoprotein (HDL), density >1.063. On occasions an "intermediate" fraction (LDL_1), of density 1.006-1.019, was also isolated. If the LDL_1 fraction was required, all 4 fractions were obtained by preparative untracentrifugation. If only the 3 major fractions were required, centrifugation and precipitation techniques were combined, as described below.

Preparation of Lipoproteins Using Ultracentrifugation. Five ml of heparinized plasma, at 4°C and density 1.006, containing 0.1-1 mM EDTA (dipotassium ethylene diamine tetra acetate) was centrifuged in cellulose acetate tubes in a Spinco L2-50 Preparative Ultracentrifuge at 39,000 rpm for 16 hours using a 40.3 fixed angle rotor (105,000 g). The supernatant containing VLDL was removed from the top by a tube slicing technique.

Any resulting pellet was redissolved in the infranatant, the volume of which was made up to 5 ml, and the density adjusted to 1.019, as described by Havel *et al* (1955). It was then centrifuged at 4°C for 18-20 hours at 39,000 rpm. The supernatant containing fraction 1.006-1.019 (LDL_1) was removed using the same slicing technique, and the 5 mls infranatant adjusted to density 1.063 by addition of potassium bromide solution. Recentrifugation at the same velocity for 20 hours resulted in a clear separation of *d* 1.019-1.063 (LDL_2) in the supernatant and HDL in the infranatant.

Isolation of Lipoproteins Using Precipitation. Following removal of VLDL by ultracentrifugation at density 1.006, LDL were precipitated from the infranatant by adding 1000 units of heparin sulphate and 0.25 ml of 1 mM manganous chloride to every 5 ml of infranatant (Fredrickson *et al*, 1967). The precipitate was centrifuged at 4°C for 20 minutes at 2,000 rpm in a refrigerated centrifuge, and the supernatant containing HDL was decanted from the pellet (LDL). The latter was suspended in normal saline.

Lipid Extraction from the Lipoproteins. Lipoprotein lipids were extracted in Dole's solution (Isopropanol:Heptane:1 N Sulphuric acid; 400:100:10) (Dole, 1956), using a ratio of 10 ml of Dole's solution for 2 ml of lipoprotein solution. Lipids were then separated from the precipitated proteins using heptane (Dole's:heptane:water; 10:6:8). Aliquots of each fraction were analyzed for cholesterol and triglyceride content using the Technicon Auto Analyser Mark II Colorimeter.

Recoveries of cholesterol and triglyceride using these techniques averaged 93 and 91% respectively. For each plasma sample the lipid content of each fraction was proportionately adjusted to 100% according to the percentage recovery for that individual sample.

3. Separation of Free and Esterified Cholesterol Samples of the lipid extracts of the lipoprotein fractions were applied to silica gel plates (0.3-0.4 mm thick) and separated using a solvent mixture of

hexane:diethyl ether:methanol:acetic acid; 180:40:6:4. The separated bands of free cholesterol and esterified cholesterol were visualized using Rhodamine G under ultraviolet light and eluted with diethyl ether. The cholesterol content was then estimated in the Auto Analyser.

RESULTS

A. PLASMA LIPID AND LIPOPROTEIN CHANGES DURING PREGNANCY

Plasma Cholesterol and Triglyceride. The plasma cholesterol level showed an overall 53% increase during pregnancy (Table 2.1, Figure 2.1), some two-thirds of which occurred within the second trimester. The plasma triglyceride concentration increased 3-fold during pregnancy. Most of this increase took place in the last trimester of pregnancy.

Plasma Lipoproteins. All 3 major lipoprotein groups increased during pregnancy.

VLDL There was no change in VLDL-cholesterol until midway through gestation, when the concentration doubled; this rose during the last trimester to a value four-times greater than normal. The ratio of free to esterified cholesterol was maintained at 1:1 throughout pregnancy (Table 2.2). VLDL-triglyceride concentration underwent approximately parallel changes, so that the triglyceride:cholesterol ratio remained constant throughout pregnancy (control 4.5:1, at delivery 4.2:1), even though the triglyceride level exceeded normal by statistical definition later than the cholesterol level (Table 2.1a).

LDL LDL-cholesterol was also unchanged at the end of the first trimester, with a cholesterol:triglyceride ratio very similar to normal (Table 2.1). However, the cholesterol concentration then increased rapidly, being 56% greater than normal at its maximal point close to term. The free to esterified cholesterol ratio decreased slightly during pregnancy from 0.42:1 in the control group to 0.35:1 during labour. The triglyceride increase in LDL was proportionately greater than that of cholesterol, so that the cholesterol:triglyceride ratio changed from greater than 3:1 in

TABLE 2.1

Plasma Lipid and Lipoprotein Changes During Pregnancy and in the Puerperium

	n	Plasma Lipid		VLDL		LDL		HDL	
		Chol.	T.G.	Chol.	T.G.	Chol.	T.G.	Chol.	T.G.
Normal†	15	202 ± 4.7*	95 ± 9.5	9 ± 1.2	45 ± 8.6	129 ± 3.9	31 ± 2.2	63 ± 3.7	20 ± 1.9
<8 wks gestation	6	200 ± 13.2	96 ± 12.1						
9-12 wks gestation	5	198 ± 5.9	123 ± 12.9						
13-18 wks gestation	9	223 ± 12.9	113 ± 8.6	10 ± 1.9	32 ± 7.8	137 ± 13.1	49 ± 4.6	76 ± 3.5	32 ± 4.4
19-24 wks gestation	10	279 ± 10.4	168 ± 20.9	18 ± 4.4	59 ± 16.0	179 ± 9.0	66 ± 7.0	81 ± 5.1	42 ± 2.9
25-30 wks gestation	13	274 ± 15.2	208 ± 18.2	20 ± 3.3	82 ± 12.8	176 ± 13.0	81 ± 5.9	79 ± 10.9	44 ± 5.9
31-36 wks gestation	23	297 ± 14.6	294 ± 23.9	39 ± 5.2	130 ± 14.7	189 ± 12.7	118 ± 11.2	67 ± 3.9	48 ± 3.1
37-40 wks gestation	25	317 ± 17.1	312 ± 22.5	37 ± 3.2	148 ± 12.6	214 ± 16.2	122 ± 11.7	66 ± 3.1	42 ± 2.2
During labour	20	303 ± 17.0	306 ± 23.9	40 ± 4.4	155 ± 13.9	190 ± 14.0	108 ± 9.8	75 ± 3.9	42 ± 2.0
Delivery	35	312 ± 13.9	296 ± 15.9	35 ± 2.7	140 ± 9.8	192 ± 10.8	109 ± 8.4	82 ± 4.5	46 ± 2.4
Day 1 post partum	23	268 ± 15.4	224 ± 19.2	26 ± 2.7	106 ± 9.0	168 ± 14.4	85 ± 9.9	75 ± 4.4	33 ± 2.5
Day 5 post partum	43	283 ± 10.9	174 ± 8.6	21 ± 1.8	79 ± 6.3	189 ± 10.8	71 ± 4.2	73 ± 2.9	25 ± 1.0
6-7 wks post partum	30	257 ± 13.2	123 ± 7.5	13 ± 1.5	49 ± 4.8	184 ± 13.1	54 ± 3.9	61 ± 3.9	21 ± 1.1
12 mths post partum	14	204 ± 11.7	117 ± 12.6	12 ± 1.8	50 ± 9.2	141 ± 10.7	47 ± 4.2	52 ± 3.6	20 ± 1.5

*Concentration: mg/100 ml (mean ± S.E.M.)

†Normal: 3rd week of menstrual cycle

FIGURE 2.1 Plasma Lipid Changes During Pregnancy and in the Puerperium

Fasting plasma cholesterol and triglyceride concentrations were measured serially throughout pregnancy, and in the puerperium and at 12 months. The results are the mean ± S.E.M. of the group, including normal and complicated pregnancies.

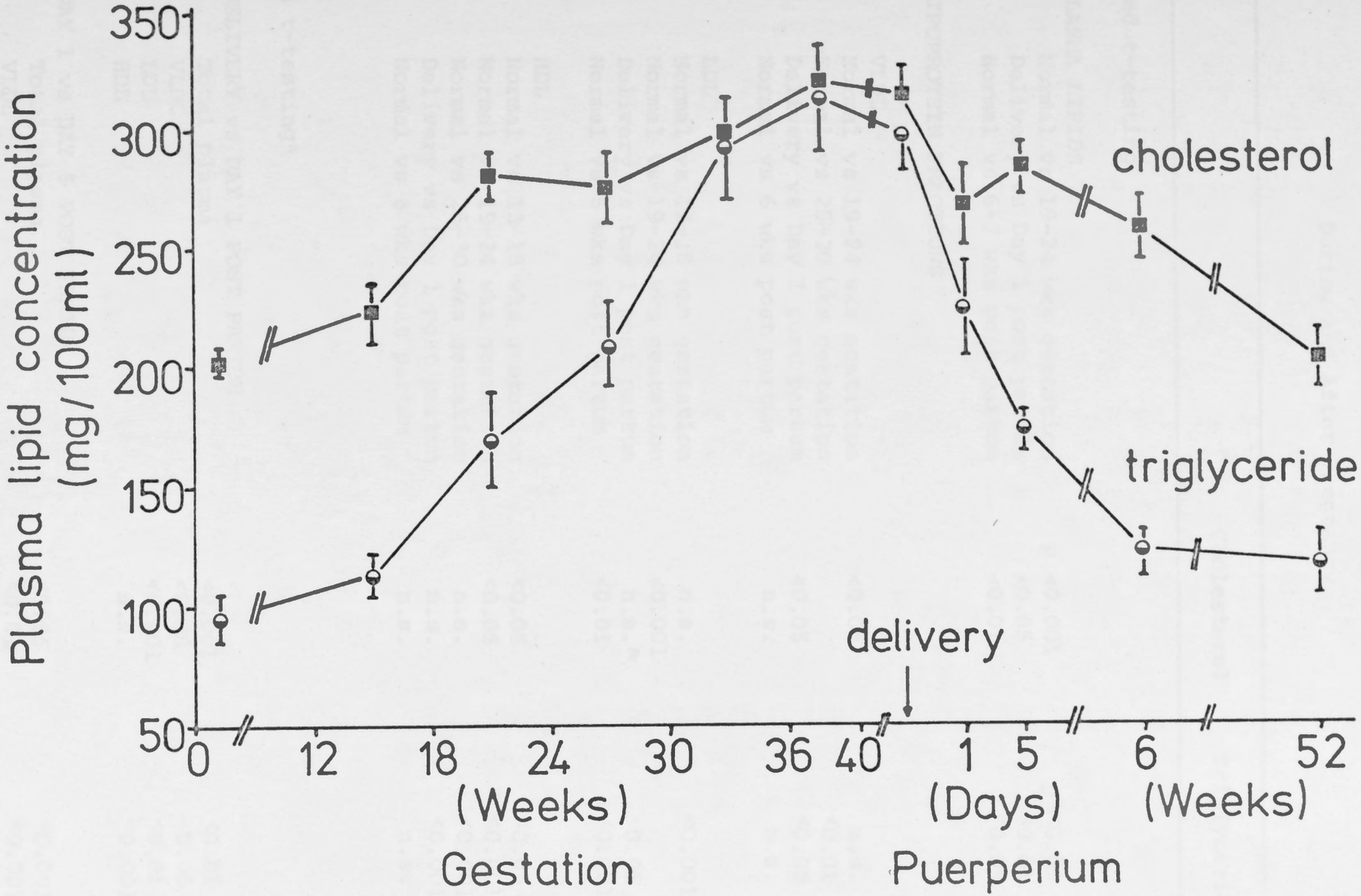

Plasma lipid concentration (mg/100 ml)
350
300
250
200
150
100
50
cholesterol
triglyceride
delivery
0
12
18
24
30
36
40
1
5
6
52
(Weeks)
(Days)
(Weeks)
Gestation
Puerperium

TABLE 2.1a

Plasma Lipid and Lipoprotein Changes During Pregnancy and in the Puerperium: Significant Differences of Some Comparisons Before, During and After Pregnancy

	Cholesterol	Triglyceride
Unpaired t-testing		
PLASMA LIPIDS		
Normal vs 19-24 wks gestation	p <0.001	p <0.01
Delivery vs Day 1 post partum	<0.05	<0.01
Normal vs 6-7 wks post partum	<0.01	<0.05
LIPOPROTEIN FRACTIONS		
VLDL		
Normal vs 19-24 wks gestation	<0.05	n.s.
Normal vs 25-30 wks gestation		<0.01
Delivery vs Day 1 post partum	<0.05	<0.05
Normal vs 6 wks post partum	n.s.	n.s.
LDL		
Normal vs 13-18 wks gestation	n.s.	<0.001
Normal vs 19-24 wks gestation	<0.001	
Delivery vs Day 1 post partum	n.s.*	0.06
Normal vs 6 wks post partum	<0.01	<0.001
HDL		
Normal vs 13-18 wks gestation	<0.05	<0.05
Normal vs 19-24 wks gestation	<0.05	<0.001
Normal vs 25-30 wks gestation	n.s.	<0.001
Delivery vs Day 1 post partum	n.s.	<0.001
Normal vs 6 wks post partum	n.s.	n.s.
Paired t-testing*		
DELIVERY vs DAY 1 POST PARTUM		
Total plasma	<0.001	<0.01
VLDL	<0.05	0.06
LDL	<0.001	<0.01
HDL	n.s.	<0.001
DAY 1 vs DAY 5 POST PARTUM		
Total plasma	<0.05	<0.001
VLDL	<0.01	<0.001
LDL	<0.001	<0.01
HDL	n.s.	<0.001

TABLE 2.2

Plasma Lipoprotein Changes During Pregnancy and in the Puerperium:

Free and Esterified Cholesterol

	n	VLDL		LDL		HDL	
		Free	Esterified	Free	Esterified	Free	Esterified
Normal†	12	5 ± 3.6*	5 ± 2.5	38 ± 5.1	89 ± 8.8	15 ± 6.9	48 ± 21.9
25-30 wks gestation	5	8 ± 4.0	10 ± 5.8	39 ± 11.1	104 ± 32.6	17 ± 5.1	53 ± 15.2
31-36 wks gestation	5	19 ± 12.2	30 ± 25.0	73 ± 39.8	171 ± 69.5	18 ± 11.5	54 ± 16.8
37-40 wks gestation	8	19 ± 7.4	24 ± 12.1	61 ± 30.0	162 ± 79.0	16 ± 5.3	49 ± 12.0
During labour	5	18 ± 12.4	22 ± 17.0	50 ± 25.2	144 ± 76.4	15 ± 3.3	54 ± 13.0
At delivery	8	14 ± 8.4	17 ± 10.4	55 ± 27.6	143 ± 58.6	19 ± 7.7	63 ± 17.4
Day 1 post partum	8	12 ± 7.7	14 ± 9.4	48 ± 28.4	123 ± 84.7	17 ± 4.9	59 ± 14.5
Day 5 post partum	12	9 ± 5.3	12 ± 8.3	48 ± 14.6	130 ± 55.3	17 ± 5.5	59 ± 15.6
6-7 wks post partum	8	7 ± 6.7	8 ± 7.1	44 ± 11.5	116 ± 28.9	13 ± 5.3	51 ± 10.6

*Concentration: mg/100 ml (mean ± S.D.)

†Normal: 3rd week of menstrual cycle

the control group to 2.2:1 late in the second trimester and 1.76:1 at delivery. When the LDL was subfractionated into 2 classes, those having densities of 1.006-1.019 (LDL_1) and of 1.019-1.063 (LDL_2), it was found that a large portion of the excess triglyceride was contained within LDL_1 (Table 2.3). The ratio of cholesterol to triglyceride in LDL_1 was maintained close to that of the normal population (0.55:1) during pregnancy, but increased to 0.68:1 at delivery. This was closer to the ratio found in VLDL rather than the LDL_2 fraction (0.20:1 and 5.2:1 respectively), and was a reflection of the heterogeneity of the total class of LDL. The ratio of cholesterol to triglyceride in LDL_2 decreased in pregnancy, but less markedly than the total LDL fraction, close to term being 2.09:1 and 1.75:1 respectively.

HDL The least dramatic increase in cholesterol concentrations occurred in the HDL fraction. The increase took place in the first trimester, but was only 21% above the level found in the third week of the normal menstrual cycle (the probable level at the time of conception). In contrast, the triglyceride component of HDL increased by 60% in the first trimester and a further 50% in the second trimester, remaining at that level until delivery. This resulted in a decrease in the cholesterol: triglyceride ratio from 3.1:1 (day 21 of the menstrual cycle, Table 2.1) to 2.4:1 early in the second trimester and 1.8:1 at delivery.

If the distribution of cholesterol and triglyceride in each fraction is expressed in terms of percentages of the total present in the plasma, the proportion of cholesterol carried in VLDL increased from 4.5% in the non-pregnant controls to 11.0% at delivery. In LDL and HDL cholesterol decreased (from 64.0% and 31.5% to 61.5% and 26.5% respectively). The percentage of total plasma triglyceride carried in VLDL was unchanged (47.5%), but increased in LDL (from 32.5% to 37.0%) and decreased in HDL (from 21.0% to 15.5%).

TABLE 2.3

Plasma Lipoprotein Changes During Pregnancy and in the Puerperium: LDL_1 and LDL_2

	n	LDL (1.006-1.063) Chol.	LDL (1.006-1.063) T.G.	n	LDL_1 (1.006-1.019) Chol.	LDL_1 (1.006-1.019) T.G.	LDL_2 (1.019-1.063) Chol.	LDL_2 (1.019-1.063) T.G.
Normal†	13	129 ± 3.9*	31 ± 2.2		5 ± 0.5	9 ± 0.9	125 ± 3.6	24 ± 1.3
13-18 wks gestation	9	137 ± 13.1	49 ± 4.6	3	4 (2- 5)**	8 (1-11)	140 (113-156)	34 (28-39)
19-24 wks gestation	10	179 ± 9.0	66 ± 7.0	8	7 ± 1.8	19 ± 3.3	175 ± 7.6	51 ± 5.8
25-30 wks gestation	13	176 ± 13.0	81 ± 5.9	7	10 ± 2.7	16 ± 3.4	161 ± 21.8	58 ± 7.8
31-36 wks gestation	23	189 ± 12.7	118 ± 11.2	9	24 ± 7.1	36 ± 6.2	158 ± 17.2	73 ± 6.7
37-40 wks gestation	25	214 ± 16.2	122 ± 11.7	8	32 ± 6.3	36 ± 4.7	205 ± 28.0	98 ± 16.8
At delivery	35	192 ± 10.8	109 ± 8.4	10	34 ± 6.6	50 ± 11.5	168 ± 27.8	90 ± 14.6
Day 1 post partum	23	168 ± 14.4	85 ± 9.9	2	24 (14-33)	28 (25-30)	121 (96-146)	43 (38-48)
Day 5 post partum	43	189 ± 10.8	71 ± 4.2	13	21 ± 4.5	21 ± 3.7	174 ± 26.9	51 ± 7.0
6-7 wks post partum	30	184 ± 13.1	54 ± 3.9	7	13 ± 6.9	14 ± 3.8	182 ± 40.8	43 ± 7.8

*Concentration: mg/100 ml (mean ± S.E.M.)

**Range

†Normal: 3rd week of menstrual cycle

B. PLASMA LIPID AND LIPOPROTEIN CHANGES IN THE PUERPERIUM

Plasma Cholesterol and Triglyceride. Within 12-24 hours of delivery, the mean plasma cholesterol had decreased from 312 mg/100 ml to 268 mg/100 ml, i.e. by 14% (Table 2.1, Figure 2.1). Measurement on day 5 post partum showed that some rebound had occurred, and at 6-7 weeks post partum, the women still had raised cholesterol levels (Table 2.1a).

Plasma triglyceride also decreased rapidly following delivery, having fallen from 296 mg/100 ml at delivery to 224 mg/100 ml within 24 hours (24%), and to 174 mg/100 ml by the fifth day. The plasma triglyceride concentration was only minimally raised 6-7 weeks after delivery (Table 2.1, 2.1a).

Plasma Lipoproteins.

VLDL Both cholesterol and triglyceride decreased markedly within the first 24 hours after delivery (26% and 24% respectively). This decrease continued over the next few weeks, so that by 6-7 weeks post partum, the lipid concentration within this fraction had returned to the non-pregnant state.

LDL As in all other fractions, the lipid content decreased following delivery; by 12% for cholesterol and 22% for triglyceride. However, the early rebound seen in the total plasma cholesterol level was totally accounted for in the LDL moiety, the cholesterol content of which, 5 days post partum, was the same as that at the time of delivery, and decreased only slightly over the next few weeks. Most of this was within the LDL_2 fraction (Table 2.3). In contrast, the triglyceride concentration decreased much more rapidly over the next few weeks on both LDL subfractions.

HDL Both lipids changed rapidly, returning to normal levels within the puerperium, following a 9% decrease in cholesterol and 28% in triglyceride within the first 24 hours.

C. INTER- AND INTRA-LIPOPROTEIN RELATIONSHIPS

It has been noted that the increase in plasma triglyceride during pregnancy was reflected by an increase in the triglyceride content of all lipoprotein fractions (Table 2.1). Part of the increase of triglyceride in the LDL fraction could be attributed to a five-fold increase in the subfraction *d* 1.006-1.019. A positive significant correlation was found for the concentration of triglyceride in these two fractions in both the non-pregnant controls and in women during the third trimester of pregnancy (Table 2.4).

TABLE 2.4

Plasma Lipoproteins: The Relationship Between the Triglyceride Concentrations of VLDL (*d* <1.006) and LDL_1 (*d* 1.006-1.019)

	n	Correlation Coefficient (r)	Significance (p)
Non-pregnant	13	0.626	<0.05
Second trimester	11	0.496	n.s.
Third trimester	16	0.844	<0.001

It has been shown that VLDL and HDL metabolism are interrelated in several ways (Margolis and Capuzzi, 1972). Some relationships were therefore sought between VLDL and HDL lipids. Figure 2.2 shows 3 sets of data; from normal, non-pregnant controls, from uncomplicated pregnancies, and from hypertensive pregnancies. There is a positive, but weak relationship between the VLDL-triglyceride concentration and the ratio of the triglyceride and cholesterol concentrations in HDL (Table 2.5).

FIGURE 2.2 Plasma Lipoproteins: The Relationship Between the Ratio of HDL Triglyceride:Cholesterol Concentrations and VLDL-Triglyceride

The ratio of the triglyceride and cholesterol concentrations in the HDL fraction in plasma samples from normal, non-pregnant women and from women at delivery was compared against the triglyceride content of VLDL from the sample sample. The correlation coefficient for the whole group was 0.536 ($p < 0.001$).

● Normal, non-pregnant women

■ At delivery, normal pregnancy

◒ At delivery, with pre-eclampsia

2·18
1·0
0·8
0·6
0·4
0·2
Ratio HDL [TG/Chol.]
50
100
150
200
250
VLDL-TG concentration (mg/100 ml)

TABLE 2.5

Plasma Lipoproteins: The Relationship Between the Ratio of HDL Triglyceride:Cholesterol Concentrations and VLDL-lipids

HDL $\left[\frac{\text{Triglyceride}}{\text{Cholesterol}}\right]$	n	VLDL-Triglyceride r	VLDL-Triglyceride p	VLDL-Cholesterol r	VLDL-Cholesterol p
Non-pregnant	13	0.381	n.s.	0.340	n.s.
Pregnant:					
Normal delivery	19	0.614	<0.01	0.584	<0.01
Hypertensive delivery	13	-0.067	n.s.	-0.065	n.s.
All subjects	47	0.536	<0.001	0.506	<0.001

The correlation was not significant in the normal, non-pregnant subjects alone, but did become highly significant at delivery and for the whole group. The above relationship also held true between VLDL-cholesterol and the HDL triglyceride:cholesterol ratio (Table 2.5).

D. FACTORS THAT MAY INFLUENCE LIPID METABOLISM DURING PREGNANCY

1. Maternal Age and Parity The plasma lipid levels at the time of delivery were analyzed in a group of 86 women, to examine the possible effects of age and parity. In neither the whole group (Table 2.6a) nor among those women who had a total uneventful pregnancy and labour (Table 2.6b) was there a significant effect on the plasma cholesterol and triglyceride levels attributable to age or to parity.

2. Pre-Existing Hypercholesterolaemia Three of the women included in the study were known to have pre-existing hypercholesterolaemia. A summary of the changes in their plasma lipids during the 3 trimesters is shown in Figure 2.3. Table 2.7 summarizes the cholesterol and triglyceride

TABLE 2.6

Plasma Lipid Changes During Pregnancy:

The Effect of Maternal Age and Parity at Delivery

(a) Among all women

Age	Parity 1	2	3	≥4
PLASMA CHOLESTEROL*				
<20 years	367 ± 14.8 (3)		290	
21-25 years	309 ± 14.8 (22)	303 ± 22.1 (12)	326 ± 38.3 (5)	298 (295-300)
26-30 years	334 ± 19.5 (7)	286 ± 10.6 (9)	369 ± 28.4 (6)	294 (288-300)
31-35 years	265 (225-305)	249	285	289 (280-298)
≥36 years				359 (328-390)
PLASMA TRIGLYCERIDE*				
<20 years	386 ± 93.0 (3)		275	
21-25 years	291 ± 20.2 (22)	281 ± 22.1 (12)	270 ± 29.5 (5)	254 (220-273)
26-30 years	270 ± 25.0 (7)	270 ± 16.4 (9)	319 ± 35.1 (6)	258 (245-280)
31-35 years	166 (155-178)	338	255	279 (220-378)
≥36 years				358 (316-400)

*Concentration: mg/100 ml (mean ± S.E.M.) (n) or (range)

TABLE 2.6

(b) Among women with a normal pregnancy and labour

Age	n	Plasma Cholesterol*	Plasma Triglyceride*
THE EFFECT OF AGE			
≤20 years	2	318 ± 27.5	273 ± 2.5
21-25 years	21	291 ± 15.9	270 ± 18.4
26-30 years	16	317 ± 10.8	284 ± 15.6
31-35 years	4	292 ± 5.8	248 ± 34.0

Parity	n	Plasma Cholesterol*	Plasma Triglyceride*
THE EFFECT OF PARITY			
1	34	317 ± 11.1	288 ± 17.1
2	22	293 ± 12.8	279 ± 13.8
3	13	340 ± 20.4	292 ± 20.1
≥4	8	310 ± 14.6	288 ± 21.3

*Concentration: mg/100 ml (mean ± S.E.M.) (n) or (range)

FIGURE 2.3 Plasma Lipid Changes During Pregnancy and in the Puerperium: The Effect of Pre-Existing Hypercholesterolaemia

The fasting plasma cholesterol and triglyceride concentrations of 3 women with pre-existing hypercholesterolaemia (2 with type IIa, and one with IIb) are shown compared to values obtained from women during normal, uncomplicated pregnancies. The concentrations in the first trimester are values up to 12 weeks' gestation, in the second trimester the mean of measurements for any individual subject between weeks 13 to 24, and up to 36 weeks for the third trimester. The puerperal value is at 6-7 weeks post partum.

● Women having normal, uncomplicated pregnancies

■ Women with pre-existing hypercholesterolaemia

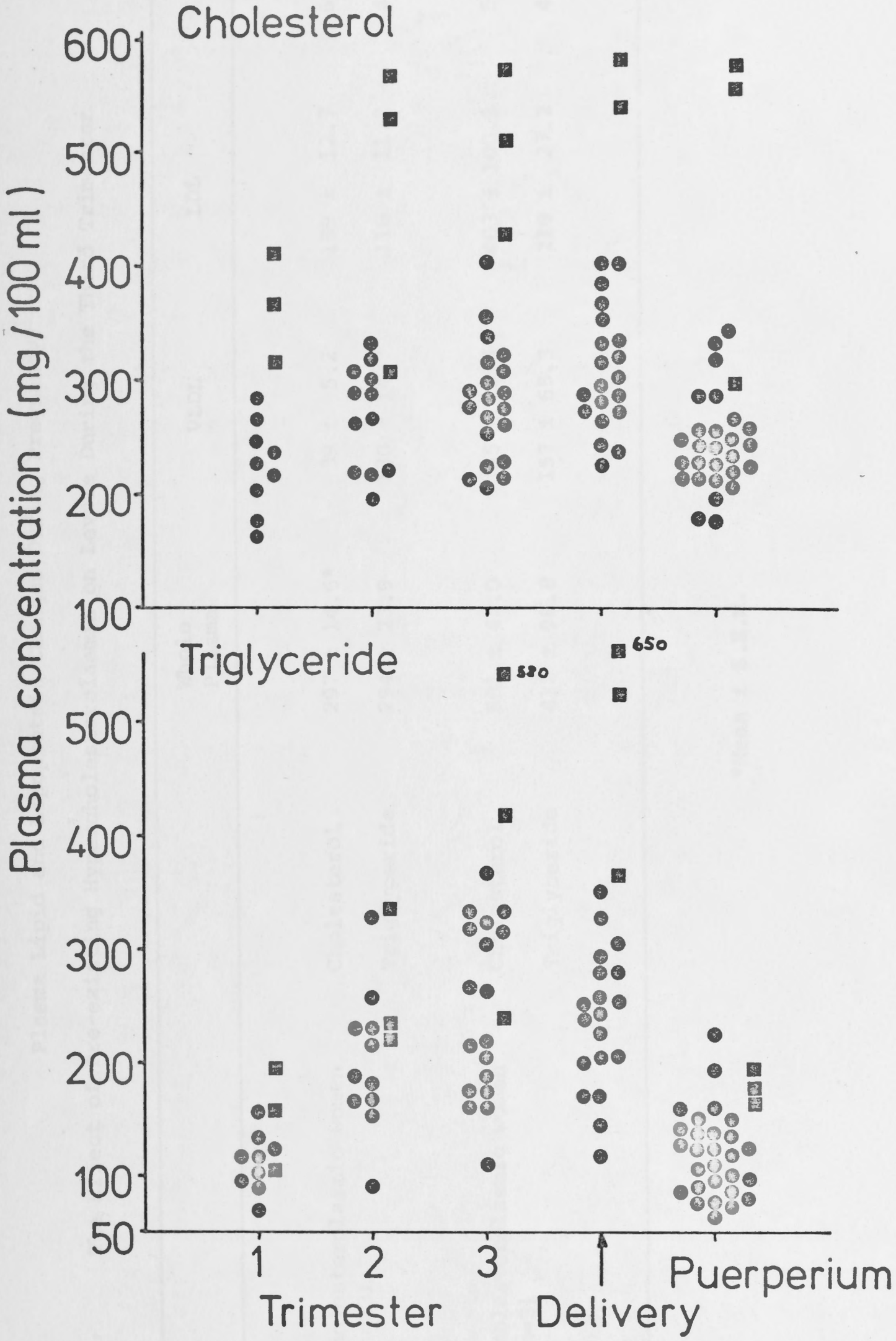

Cholesterol
Triglyceride
Plasma concentration (mg / 100 ml)
600
500
400
300
200
100
50
520
650
1
2
3
Trimester
Delivery
Puerperium

TABLE 2.7

Plasma Lipid and Lipoprotein Changes During Pregnancy:

The Effect of Pre-existing Hypercholesterolaemia on Levels During the Third Trimester

		Whole Plasma	VLDL	LDL	HDL
Normocholesterolaemic women (n=23)	Cholesterol	297 ± 14.6*	39 ± 5.2	189 ± 12.7	67 ± 3.9
	Triglyceride	294 ± 23.9	130 ± 14.7	118 ± 11.2	48 ± 3.1
Hypercholesterolaemic women (n=3)	Cholesterol	501 ± 42.0	45 ± 18.5	403 ± 100.5	53 ± 2.5
	Triglyceride	412 ± 98.8	157 ± 65.3	219 ± 37.2	42 ± 7.5

*Mean ± S.E.M.

concentrations of the lipoprotein fractions during the third trimester. The only treatment during pregnancy was a low cholesterol diet in one individual. The percentage increase in whole plasma cholesterol and triglyceride at delivery was of the same order as seen in the total population, plasma cholesterol being 39%, 69% and 51% greater and plasma triglyceride 2.70-, 4.10- and 2.29-fold greater respectively.

The absolute amount of cholesterol and triglyceride within VLDL in the third trimester was only slightly above that found for the total population (Table 2.7), whilst the cholesterol content of HDL was significantly less ($p < 0.05$). The large increase in cholesterol and triglyceride occurred in the LDL fraction, the ratio of cholesterol to triglyceride being similar to that in the total pregnant population (1.84:1 and 1.60:1 respectively).

Clinically, the tendinous xanthomata of one subject showed a marked increase in size from early in the second trimester. These appear to be slowly regressing 6 months after delivery. Prominent arcus senilis also developed early in pregnancy. By 6-8 weeks post partum this had regressed and disappeared.

3. Hypertension and Pre-Eclampsia Hypertension is not uncommonly recorded during labour. In the event of this occurring there is a difference in both whole plasma lipids and in the lipoprotein distribution (Table 2.8). For the purposes of this analysis, the control group consisted of all samples obtained from normal deliveries in women who had an uncomplicated pregnancy. Cases of pre-existing hypercholesterolaemia have been excluded from both groups. The hypertensive group included all women in whom a recording of hypertension ($\geq$140/90) was made during labour, within 6 hours of delivery, and women with essential hypertension throughout pregnancy. The hypertensive group consisted of 3 women in whom the diagnosis of pre-eclampsia had been made (1 severe), 3 women with essential hypertension and 7 women who developed hypertension in

TABLE 2.8

Plasma Lipid and Lipoprotein Changes During Pregnancy:

The Effect of Hypertension and Pre-Eclampsia

		Whole Plasma	VLDL	LDL	HDL
AT DELIVERY					
Normotensive women (n=19)	Cholesterol	299 ± 12.1*	27 ± 2.4	184 ± 8.5	87 ± 6.3
	Triglyceride	237 ± 14.2	109 ± 10.5	90 ± 4.6	39 ± 1.7
Hypertensive women (n=13)	Cholesterol	308 ± 16.7	43 ± 3.5	186 ± 15.7	71 ± 6.5
	Triglyceride	327 ± 15.7	167 ± 14.2	108 ± 7.7	50 ± 2.9
Significant differences (p)	Cholesterol		<0.001		
	Triglyceride	<0.001	<0.01	<0.05	<0.01
DURING PREGNANCY (31-40 wks)					
Normotensive women (n=12)	Cholesterol	274 ± 15.0	24 ± 3.5	170 ± 13.4	75 ± 4.0
	Triglyceride	231 ± 21.1	112 ± 15.0	84 ± 7.3	35 ± 2.4
Hypertensive women (n=7)	Cholesterol	307 ± 21.8	43 ± 5.1	198 ± 20.9	64 ± 7.1
	Triglyceride	332 ± 20.6	171 ± 20.9	115 ± 9.7	47 ± 3.4
Significant differences (p)	Cholesterol		<0.01		
	Triglyceride	<0.01	<0.05	<0.05	<0.01

*Concentration: mg/100 ml (mean ± S.E.M.)

labour. In one of the latter, the diagnosis of eclampsia was made retrospectively following an abrupt increase in blood pressure and a post-partum haemorrhage. The strict definition of pre-eclampsia should include all subjects who develop hypertension during labour, as well as those diagnosed during gestation on grounds of weight, hypertension and oedema (Baird, 1969).

As mentioned in the methods section, samples taken during labour and at delivery are technically non-fasting, but there was no statistical difference in plasma lipid concentrations between these two groups (i.e. during labour and at delivery) and that taken in the fasting state at 37-40 weeks of gestation (Table 2.1).

As can be seen from the data in Table 2.8, the plasma triglyceride concentrations at delivery of the normal and hypertensive women were significantly different. There was no difference in total cholesterol levels. This was confirmed among the larger group of women (n=86), from whom data for age and parity were extracted. The excess plasma triglyceride occurred mainly in the VLDL, with a corresponding increase in cholesterol, so that the triglyceride:cholesterol ratio was maintained at approximately 4:1. The absolute amount of LDL-cholesterol was similar in the two groups, but there was a significant excess of triglyceride in the hypertensive groups. The LDL_1 subfraction also reflected this, the cholesterol and triglyceride concentrations being 20 mg/100 ml and 25 mg/100 ml respectively in the normals compared with 26 and 35 mg/100 ml in the hypertensive groups. In the hypertensive women the HDL-triglyceride content was increased, but cholesterol decreased.

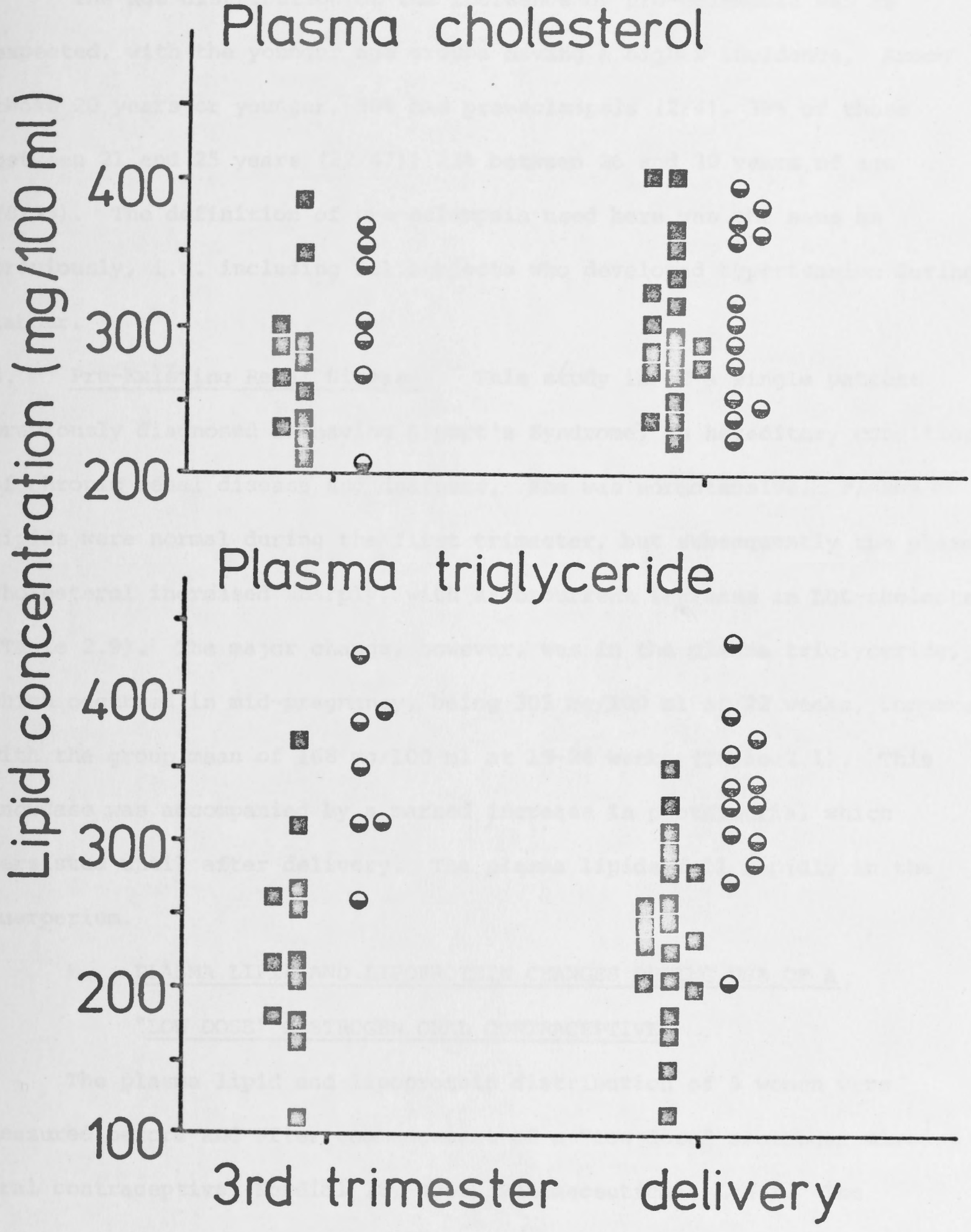
In the normal and hypertensive women, lipid measurements were also made during the third trimester of pregnancy in about half. These are shown in Figure 2.4 and Table 2.8. The higher triglyceride values were already present in the hypertensive group, as was the difference in lipoprotein distribution. In the puerperium there was no statistical

FIGURE 2.4 Plasma Lipid Changes During Pregnancy: The Effect of Hypertension and Pre-Eclampsia

The fasting plasma cholesterol and triglyceride concentrations found in women who already had hypertension, or developed hypertension and pre-eclampsia are compared with values found in women who had uncomplicated pregnancies. The third trimester values are measurements made between weeks 31-36 of gestation.

■ Women with normal, uncomplicated pregnancies

◒ Women who had hypertension and/or pre-eclampsia


Plasma cholesterol
Plasma triglyceride
Lipid concentration (mg/100 ml)
400
300
200
400
300
200
100
3rd trimester
delivery

difference between the triglyceride levels in the hypertensive and normal groups.

The age distribution of the incidence of pre-eclampsia was as expected, with the younger age groups having a higher incidence. Among those 20 years or younger, 50% had pre-eclampsia (2/4), 39% of those between 21 and 25 years (22/47), 23% between 26 and 30 years of age (8/33). The definition of pre-eclampsia used here was the same as previously, i.e. including all subjects who developed hypertension during labour.

4. Pre-Existing Renal Disease This study is of a single patient previously diagnosed as having Alport's Syndrome, an hereditary condition of chronic renal disease and deafness. She was normotensive. Plasma lipids were normal during the first trimester, but subsequently the plasma cholesterol increased sharply, with a concurrent increase in LDL-cholesterol (Table 2.9). The major change, however, was in the plasma triglyceride, which occurred in mid-pregnancy, being 305 mg/100 ml at 22 weeks, compared with the group mean of 168 mg/100 ml at 19-24 weeks (Table 2.1). This increase was accompanied by a marked increase in proteinuria, which persisted until after delivery. The plasma lipids fell rapidly in the puerperium.

E. PLASMA LIPID AND LIPOPROTEIN CHANGES DURING USE OF A "LOW DOSE" OESTROGEN ORAL CONTRACEPTIVE

The plasma lipid and lipoprotein distribution of 5 women were measured before and after commencement of a "low dose" oestrogen mixed oral contraceptive (Nordiol 28, Wyeth Pharmaceuticals Ltd.). The pre-treatment sample was taken in the third week of the menstrual cycle prior to commencing treatment, and the treatment sample during the third week of the first cycle on the contraceptive preparation.

Table 2.10 shows the plasma lipid and lipoprotein distributions. Whilst the change in plasma cholesterol was not statistically significant,

TABLE 2.9

Plasma Lipid and Lipoprotein Changes During Pregnancy:

The Effect of Pre-existing Renal Disease†

		Whole Plasma	VLDL	LDL	HDL
7 weeks gestation	Cholesterol*	224	12	149	62
	Triglyceride*	140	5	63	23
14 weeks gestation	Cholesterol	280	9	196	75
	Triglyceride	158	59	69	29
22 weeks gestation	Cholesterol	318	36	205	77
	Triglyceride	305	154	96	54
36 weeks gestation	Cholesterol	415	49	258	104
	Triglyceride	387	167	160	60
At delivery	Cholesterol	537	43	293**	100
	Triglyceride	543	112	329**	101
6 wks post partum	Cholesterol	236	10	163	72
	Triglyceride	135	57	55	23

*Concentration: mg/100 ml

**

	Cholesterol	Triglyceride
LDL_1	77	140
LDL_2	216	189

†One subject only

the increase in triglyceride concentration was. Few of the lipoprotein changes achieved the level of statistical significance. In LDL_1 (*d* 1.006-1.019), both cholesterol and triglyceride concentrations increased ($p < 0.001$ and $p < 0.01$ respectively). The cholesterol concentration of HDL decreased ($p < 0.001$).

DISCUSSION

A. PLASMA LIPID AND LIPOPROTEIN CHANGES DURING PREGNANCY

Normal Pregnancy. Both plasma cholesterol and triglyceride concentrations increased markedly during pregnancy. The major increase in cholesterol occurred during the second trimester, becoming maximal during the 31st-36th weeks of gestation, remaining high until delivery. This pattern is similar to that reported by De Alvarez *et al* (1959) and Pantelakis *et al* (1964b), in contrast to descriptions of either a continued increase up to term (Moses *et al*, 1952; Vernet and Smith, 1961; Svanborg and Vikrot, 1965a) or a slight decrease prior to term (Oliver and Boyd, 1955; Watson, 1957; Hashmi and Afroze, 1972). The studies were not commenced early enough during gestation to determine the earliest changes in plasma lipids, having in mind the decreases in plasma cholesterol which have been reported during the first trimester (De Alvarez *et al*, 1959; Green, 1966). The small sample in Table 2.1 showed no decrease.

Plasma triglyceride levels became maximal in the third trimester and remained elevated until delivery. Other workers have also demonstrated a 2 to 3-fold increase (Cramér *et al*, 1964/5; Konttinen *et al*, 1964; Svanborg and Vikrot, 1965a; Renkonen, 1966; Damiani *et al*, 1972; Knopp *et al*, 1973). Svanborg and Vikrot (1965a) found a positive correlation between plasma triglyceride and time of gestation.

The lipoprotein changes during pregnancy varied in the different fractions. A significant increase in the triglyceride content of LDL had occurred by the 13th-18th weeks of gestation and in the cholesterol content

TABLE 2.10

Plasma Lipid and Lipoprotein Changes During the First Cycle on a "Low Dose" Oestrogen Mixed Oral Contraceptive

			Cholesterol Concentration*					Triglyceride Concentration*				
			Whole Plasma	VLDL	LDL_1	LDL_2	HDL	Whole Plasma	VLDL	LDL_1	LDL_2	HDL
Subject 1	Before		191	3	3	106	80	80	19	7	24	28
	After		205	6	6	131	62	92	36	13	26	15
	Δ		+14	+3	+3	+25	-18	+12	+17	+6	+ 2	-13
Subject 2	Before		205	2	4	131	67	35	8	3	14	10
	After		216	6	6	159	45	56	16	9	19	12
	Δ		+11	+4	+2	+28	-22	+21	+ 8	+6	+ 5	+ 2
Subject 3	Before		227	16	6	138	63	136	82	11	23	21
	After		262	13	12	177	59	136	58	18	40	20
	Δ		+35	-3	+6	+39	- 4	0	-24	+9	+17	- 1
Subject 4	Before		228	9	4	133	82	119	61	12	25	20
	After		199	14	7	119	59	142	81	15	25	21
	Δ		-29	+5	+3	-14	-23	+23	+20	+3	0	+ 1
Subject 5	Before		199	10	4	129	56	106	57	11	22	16
	After		194	15	7	136	36	109	62	10	23	13
	Δ		- 5	+5	+3	+ 7	-20	+ 3	+ 5	-1	+ 1	- 3
Group	Before	mean	210	8	4	127	70	95	45	9	22	19
		S.D.	16.7	5.7	1.1	12.9	11.1	39.4	30.9	3.8	4.4	6.6
	After	mean	215	11	8	144	52	107	51	13	27	16
		S.D.	27.4	4.4	2.5	23.3	11.2	35.0	25.1	3.7	8.0	4.1
Significant difference (paired t-test)			n.s.	0.06	<0.001	0.06	<0.001	0.01	n.s.	<0.01	n.s.	n.s.

*Concentration: mg/100 ml

by the 19th-24th weeks. The cholesterol and triglyceride of HDL were significantly higher during the first half of pregnancy than in non-pregnant women, but while the triglyceride component remained elevated throughout pregnancy, the cholesterol content of HDL decreased to that found in non-pregnant women. Because of the wide range of values in VLDL composition, no significant increase in VLDL cholesterol or triglyceride was found until mid-pregnancy. There are no figures available for comparison of the cholesterol and triglyceride content of lipoprotein fractions prior to the third trimester of pregnancy. The change in the ratio of β and α lipoproteins on electrophoresis during pregnancy (Oliver and Boyd, 1955; Russ *et al*, 1954; von Studnitz, 1955) and the presence of an increasing quantity of lipid in the pre-β lipoprotein band, which corresponded with VLDL on ultracentrifugation (Pantelakis *et al*, 1964b), are in accord with this study and that of Knopp *et al* (1973). During the third trimester of pregnancy, the latter authors found the triglyceride content of the 3 major lipoproteins to be increased, while the cholesterol content of VLDL and LDL was also increased.

Complications of Pregnancy. In this current study, the occurrence of hypertension and pre-eclampsia resulted in an increase in whole plasma triglyceride concentration, which was reflected mainly as an increase in VLDL. Similarly, in the case of pre-existing renal disease, the triglyceride increase was greater than in the normal population. Hypercholesterolaemia magnified the hypercholesterolaemic response. In studies of the effect of complications on plasma lipid changes, diabetic pregnancy and pre-eclampsia are two factors to have been considered. In pre-eclampsia, plasma cholesterol levels have risen higher than in normal pregnancy in some studies, though the increase was not always significant (Boyd, 1934a,b; De Alvarez and Bratvold, 1961; Konttinen *et al*, 1964). Plasma triglycerides also tend to be higher (Nelson *et al*, 1966). The

lipid changes during diabetic pregnancies have been shown to be greater than in normal pregnancies, with an increased β-band on electrophoresis evident from early in pregnancy (Vernet and Smith, 1961; Pantelakis *et al*, 1964a), and a pre-β band which may remain elevated for up to 2 weeks post partum (Vernet and Smith, 1961). Knopp *et al* (1973) have reported the plasma triglyceride level to be significantly higher during the third trimester in a group of diabetics.

Hyperlipoproteinaemic Phenotype. Considering the distribution of lipid among the lipoproteins during the third trimester, the elevation of plasma cholesterol was distributed between VLDL and LDL, whilst triglyceride was increased in all 3 fractions. The situation earlier in pregnancy differed only in that HDL cholesterol was also raised. This pattern (reported also by Knopp *et al*, 1973) conforms most closely to that of genetic combined hyperlipoproteinaemia (Rose *et al*, 1974), which may or may not correspond to Type IIb hyperlipoproteinaemia (W.H.O., 1972) and mixed hyperlipoproteinaemia (Brown *et al*, 1973). In this phenotype there is elevation of both plasma cholesterol and triglyceride with elevations of both VLDL and LDL. Such a combined pattern has been described in other metabolic disorders, notably in the nephrotic syndrome (Baxter *et al*, 1960), amongst monotropic growth hormone-deficient dwarfs (Merimee *et al*, 1972) and in insulin-deficient diabetics (Chance *et al*, 1969; Wilson *et al*, 1970). In nephrosis, the higher the VLDL increase, the less dramatic the LDL rise (Baxter *et al*, 1960) and vice versa; VLDL and HDL are also reciprocally related. Reciprocal relationships between VLDL and LDL have also been noted during weight reduction, carbohydrate induction of hypertriglyceridaemia and during clofibrate treatment (Havel and Gordon, 1960; Strisower *et al*, 1968; Nichols, 1969; Wilson and Lees, 1972), and between VLDL and HDL during carbohydrate induction (Levy *et al*, 1966) and clofibrate treatment (Strisower *et al*, 1968).

The Role of Hormones. It is likely that the hyperlipidaemia of pregnancy is related to hormonal changes.

Insulin The similarity in lipoprotein patterns between pregnancy and the insulin-deficient diabetic and the growth hormone-deficient dwarf may seem almost paradoxical. The level of insulin is elevated in both the fasting state and in response to glucose during the third trimester of pregnancy (Spellacy *et al*, 1963; Bleicher *et al*, 1964). It has been suggested that this increase is secondary to the raised free fatty acid levels leading to relative insulin resistance (Spellacy *et al*, 1963; Bleicher *et al*, 1964; Freinkel, 1964).

HPL and HGH Human placental lactogen (HPL or human chorionic sommatotrophin), which was identified in the early 1960's by several workers including Josimovich and MacLaren (1962) has chemical, immunological and biological similarities to human growth hormone (HGH), whilst also having lactogenic activity (Liggins, 1972). Its presence in the maternal circulation may be detected from the sixth week of pregnancy, and it steadily increases to become maximal at about 34 weeks (Beck and Daughaday, 1967), remaining high until delivery. Its secretion throughout pregnancy is thought to be a direct function of placental weight (Josimovich, 1971). Similar to its counterpart, HGH, it has diabetogenic effects, being glucose-sparing and resulting in mobilization of free fatty acid (Grumbach *et al*, 1968). Enhancement of *in vitro* lipolysis in adipose tissue has been demonstrated (Turtle and Kipnis, 1967) as has synergistic action with HGH (Turtle and Kipnis, 1967; Murakawa and Raben, 1968), the latter having 10 to 1000 times the activity of HPL. HPL has been demonstrated to have an insulogenic activity, increasing circulating insulin levels when infused into normal subjects (Beck and Daughaday, 1967) and producing in animals changes in β cells similar to those seen during pregnancy (Malaisse *et al*, 1969).

Oestrogens Oestrogens may also be implicated although some of the effects of oestrogens could result from the interaction with other hormones. The use of oral contraceptive preparations has been accompanied by changes in insulin levels and reactivity (Spellacy *et al*, 1967, 1968; Yen and Vela, 1968; Hazzard *et al*, 1969; Wynn and Doar, 1969), and in growth hormone levels (Spellacy, 1967a,b). Beck and Wells (1969) have suggested that oestrogens themselves may induce end-organ resistance to glucose transport in the peripheral tissues. The progestogenic components of oral contraceptives have also been implicated in changes in carbohydrate metabolism in monkeys (Beck, 1969).

Corticosteroids Plasma corticosteroids increase progressively throughout pregnancy (Bayliss *et al*, 1955). The level of free cortisol in plasma is either normal or elevated and the transcortin concentration is increased (Doe *et al*, 1969; O'Connell and Walsh, 1969). Clinically the pregnant woman shows several features of Cushing's syndrome; resistance to insulin, lymphopenia, eosinopenia, amino aciduria and glucosuria (Steinbeck, 1972). Glucocorticoids decrease glucose utilization in muscle and adipose tissue, accelerate gluconeogenesis and increase circulating free fatty acids. Increases in all lipids and lipoproteins have been observed in subjects treated with cortisone (Adlersberg *et al*, 1950, 1951).

Thyroid Function Plasma protein-bound iodine and total thyroxine increase during the first trimester and remain elevated until 6 weeks post partum (Ibbertson, 1972). Free thyroxine may be increased during the first 11-18 weeks (Fisher *et al*, 1970). Other functions of thyroid activity also alter, with the BMR increasing 20-30% by the third trimester (Freedberg *et al*, 1970). The addition of increased circulating thyroxine may further aggravate the insulin resistance of the pregnant woman. This may tend to negate the effect of increasing thyroxine, which frequently results in low levels of LDL and cholesterol (Fredrickson *et al*, 1967).

Renal Physiology and Disease. The hyperlipidaemia of pregnancy and that seen in some cases of the nephrotic syndrome show other similarities. Renal function is altered in normal pregnancies, with an increase in glomerular filtration rate and renal blood flow (Nanra and Kincaid-Smith, 1972), the former secondary to increased plasma volume. Aldosterone production is increased, as is renin-angiotensin activity. Urinary catecholamine excretion is not altered during normal pregnancy (Zuspan, 1970) but a reported sensitivity to catecholamines occurs in complications such as oedema, proteinuria and hypertension, i.e., pre-eclampsia (Raab *et al*, 1956; Talledo *et al*, 1968).

The syndrome of nephrosis is defined as hyperlipidaemia, hypoalbuminaemia, proteinuria and generalized oedema. It is then a quartet of symptoms, representative of a group of renal diseases and pathologies. Comparison of the symptoms with those that might occur during the third trimester reveal a shared hypoalbuminaemia (in pregnancy secondary to an increase in plasma volume), generalized oedema, which occurs in 30% of normal pregnancies (Thomson *et al*, 1967) and of course, hyperlipidaemia. Proteinuria in pregnancy occurs in pre-eclampsia. Hypertriglyceridaemia was more severe among the women who had hypertension at term, and who by Baird's criteria would be considered to have pre-eclampsia at delivery. Pathological renal lesions accompanying pre-eclampsia have been described (Robbins, 1967), and on occasion may be extremely difficult to differentiate from changes which occur in many of the renal diseases which produce the nephrotic syndrome. Experimental nephrosis produced in rats by anti-kidney serum is accompanied by hypoalbuminaemia, hepatic hyperplasia and marked increases in VLDL and LDL synthesis in the perfused liver (Marsh and Drabkin, 1960) or liver slices (Radding and Steinberg, 1960), as well as lipid accumulation within the liver (Hoak *et al*, 1968). The turnover of plasma esterified cholesterol and triglyceride has been reported to be increased in subjects with

nephrosis (McKenzie and Nestel, 1968; Kekki and Nikkilä, 1971b).

Lipoprotein and Lipid Metabolism. The hyperlipidaemia of pregnancy may be discussed in terms of alterations in synthesis and catabolism of lipoproteins and their lipid constituents.

Free fatty acids stimulate the hepatic secretion of triglycerides by increasing both the number and the size of the VLDL molecules. Nestel and Steinberg (1963) and Heimberg *et al* (1965) reported this relationship with liver perfusion studies. In pregnancy, increased lipolytic activity could occur as the result of HPL and increased corticosteroids.

Endogenous cholesterol synthesis increases with body weight (Nestel *et al*, 1969). The weight accumulated during a normal pregnancy is greater than that due to the increased size of the uterus and its contents and the increase in plasma volume. There is also increased deposition of body fat (Hytten and Leitch, 1972). Plasma cholesterol concentration need not be related to the rate of production or the amount of cholesterol in the two exchangeable pools in normal subjects or in individuals with established hyperlipoproteinaemia (Nestel *et al*, 1969). This does not exclude such a relationship when the plasma cholesterol is rising progressively as in pregnancy.

With respect to the protein components of the plasma lipoproteins, the half-life of LDL protein in normal women (Walton *et al*, 1963, 1965) may be shorter than in men (Gitlin *et al*, 1958; Langer *et al*, 1969), a comparison similar to that reported by Scott and Hurley (1969) who undertook longer studies. However, Langer *et al* (1972) were unable to demonstrate any sex difference. Among pathological conditions which may have some comparable features to changes during pregnancy, the turnover of LDL is apparently increased among nephrotic children (Gitlin *et al*, 1958) and in some patients with hyperlipaemia (Walton *et al*, 1965; Scott and Winterbourn, 1967). This area is clouded by the study of patients with

different forms of hyperlipidaemia. The catabolic rate of LDL has been reported to be decreased in subjects with Type IIa hyperlipoproteinaemia (Langer *et al*, 1969a,b, 1972), though Scott and Winterbourn (1967) have shown that the absolute turnover may be increased. Recent reports by Simons *et al* (1973) show an increased turnover of LDL protein in homozygous hypercholesterolaemia. Reinjected HDL-protein clearance is increased in patients with endogenous hypertriglyceridaemia, but this may be due to their diminished HDL pool (Furman *et al*, 1964). The labelled small molecular weight polypeptides of human VLDL transfer to HDL both *in vitro* and *in vivo*, while the apolipoprotein of LDL (apoLDL) found in VLDL moves unidirectionally from VLDL (d <1.006) to LDL (d 1.019-1.063) via an intermediate density range (1.006-1.019) (Bilheimer *et al*, 1972; Eisenberg *et al*, 1973). The small polypeptide transfer also occurs following infusion of human VLDL into the squirrel monkey (Schonfeld *et al*, 1972). Infusion of heparin affects the *in vivo* distribution of the radioactivity associated with the small polypeptides and their movements between VLDL and HDL, as well as in the subfractions of VLDL (Eisenberg *et al*, 1973).

Some interrelationships between lipoproteins have been briefly demonstrated in the results section. Some of these are functionally important: the catabolism of VLDL probably requires activity of the enzyme lecithin:cholesterol acyl transferase (LCAT), which initially esterifies cholesterol in HDL (Glomset, 1968). In these current studies, the ratio of free:esterified cholesterol in LDL decreased slightly from 0.42 in the non-pregnant population to 0.34 during labour. The ratios in VLDL and HDL were unchanged. Svanborg and Vikrot (1965c) were unable to demonstrate any difference in the percentage of cholesterol esterified *in vitro* in the plasma of pregnant patients. Whilst this was not a direct measure of LCAT activity, they interpreted this as showing that enzyme activity was unchanged during the third trimester. In rats, administration

of a mixed steroid contraceptive increases the rate of plasma cholesterol esterification (Aftergood *et al*, 1968).

The reason for the frequently observed reciprocal relationship between VLDL and other lipoproteins in some hyperlipaemias is unknown, but may reflect diminished catabolism of VLDL since some and possibly all LDL protein is derived from VLDL. LDL and HDL are also reciprocally related and this can be shown by treatment with oestrogens and androgens (Oliver and Boyd, 1954, 1956a,b; Hood, 1959; Robinson and Le Beau, 1965). The former results in a decreased LDL and an increased HDL concentration, VLDL also increasing, whilst the latter gives rise to the reverse. Combined preparations result in intermediate responses that depend on the relative proportions of the component steroids (Stokes and Wynn, 1971).

Triglyceride kinetics are altered by the steroids, synthesis being increased by the oestrogenic component and clearance by the progestogenic drug (Kekki and Nikkilä, 1971a; Kissebah *et al*, 1973). By extrapolation it may be considered that a similar state exists during pregnancy. The maintenance of a constant cholesterol to triglyceride ratio in VLDL in this study would support the theory of increased VLDL synthesis secondary to increased triglyceride synthesis. Increased synthesis of triglyceride in response to oestrogen treatment occurs in the absence of elevated free fatty acid levels or an increase in free fatty acid turnover (Kissebah *et al*, 1973). Plasma free fatty acid levels are raised in pregnancy (Nelson *et al*, 1966; Fioretti *et al*, 1970; Becker *et al*, 1971) and may provide an additional stimulus for greater triglyceride production. Post-heparin lipolytic activity (PHLA), the key enzyme responsible for triglyceride clearance, has been found to be depressed during pregnancy (Meng and McGanity, 1958; Fabian *et al*, 1968). If this were the case, then the basis of the hypertriglyceridaemia of pregnancy might involve reduced clearance as well as increased production. However, in the current studies, the absolute amount of LDL-cholesterol and triglyceride increased

markedly, which, together with the evidence from oral contraceptive studies, would suggest that a decrease in the rate of catabolism of VLDL was not the major cause of the hypertriglyceridaemia, although the rate of clearance was obviously not sufficient to prevent the rise in triglyceride. The reportedly low levels of PHLA did not take into account the expanded plasma volume (Hytten and Leitch, 1972). While PHLA may therefore not be low in normal pregnancy it is reduced in pregnancy complicated by diabetes (Knopp and Arky, 1972). In this latter situation there is an exaggeration of the increased triglyceride concentration of pregnancy.

B. PLASMA LIPID AND LIPOPROTEIN CHANGES DURING THE PUERPERIUM

Plasma cholesterol and triglyceride concentrations fall during the first 24 hours after delivery. The plasma triglyceride continued to decrease, but was still higher than in the non-pregnant population 6-7 weeks post partum. By comparison the plasma cholesterol concentration underwent a further increase, the LDL-cholesterol content being the same on day 5 post partum as at delivery. It subsequently decreased slowly, but was still significantly higher than in the controls at 6-7 weeks after delivery.

Previous reports have also indicated that the levels of cholesterol and triglyceride decrease in the puerperium, the cholesterol more slowly than the triglyceride (Watson, 1957; De Alvarez *et al*, 1959; Konttinen *et al*, 1964; Svanborg and Vikrot, 1965b). One report however showed some variance with the generally reported pattern of cholesterol change. Oliver and Boyd (1955) found the level to decrease after delivery, but subsequently to rise so that at the end of the first week, the plasma level was the same as, or slightly higher than, the concentration at delivery. This is similar to the findings in the present study. Since the secondary increase occurred in the LDL fraction, it probably resulted from the rapid catabolism of VLDL and conversion to LDL. The fall in VLDL

and triglyceride concentrations may have partly reflected the fall in free fatty acid concentration, which occurs after delivery of the placenta (Fairweather, 1965), and resembles the rapid fall seen after commencing nicotinic acid (Carlson *et al*, 1968). The kinetics of free fatty acid and triglyceride in VLDL are much more rapid than that of cholesterol, and the more rapid fall in triglyceride than in cholesterol would be expected. The turnover of free fatty acid is measured in minutes, that of triglyceride in hours, but that of cholesterol in weeks.

Both cholesterol and triglyceride were significantly higher than in the control women 6-7 weeks post partum. This finding was similar to that of other workers, who have found lipid levels to remain elevated for weeks or months after cessation of pregnancy (Peters *et al*, 1951; Oliver and Boyd, 1955; De Alvarez *et al*, 1959). Lactation did not apparently influence lipid levels. The post-partum hyperlipidaemia was found to be very sensitive to dietary manipulation, responding quickly to changes in dietary cholesterol and fatty acid composition (Chapter 6). This is in contradistinction to findings during pregnancy, when diet does not readily influence the hyperlipidaemia (Moses *et al*, 1952; Hansen *et al*, 1964; Mullick *et al*, 1964; Green, 1966). The persistent post-partum hyperlipidaemia might therefore reflect dietary factors. The possibility that it may be hormonally mediated is supported by the findings that hyperlipidaemia may persist after use of sex steroid preparations, e.g. oral contraceptives. The altered triglyceride kinetics may be detected up to 6 weeks after stopping their use (Kekki and Nikkilä, 1971a). Many physiological parameters apparently return to normal very quickly after removal of the foetus and placenta; for instance there is an immediate improvement in the hypertension of pre-eclampsia and eclampsia, though no difference was found in the rate of lipid change among this group of women as compared with the others. Patients suffering from cholestasis of pregnancy or that due to the use of oral contraceptives rapidly lose

symptoms such as pruritis caused by the retention of bile salts (Adlercreutz *et al*, 1968). Hepatic excretory function which may be diminished during pregnancy as measured by bromsulphthalein excretion (e.g. Combes *et al*, 1963) improves rapidly post-partum as shown by the increased biliary concentration of cholesterol and bile acids in the first week after delivery (Large *et al*, 1960). However, the restoration of other metabolic functions and the return of hormonal equilibrium to the non-pregnant state might conceivably take much longer.

CHAPTER 3

STEROL AND BILE ACID EXCRETION DURING PREGNANCY

INTRODUCTION

The hyperlipaemia of pregnancy conforms most closely to the phenotype of combined hyperlipoproteinaemia. The plasma cholesterol concentration increases significantly and maximally during the second trimester, while the increase in plasma triglyceride is maximal in the third trimester. Possible differences in the handling of cholesterol and bile acids may be partly responsible for, or related to, the hyperlipidaemia.

The incidence of gall-bladder disease is higher among women of childbearing age than among men of the same age (Kaye and Kern, 1971). The secretion of biliary cholesterol is similar among men and women, but bile acid secretion may be reduced in some women (Grundy *et al*, 1972). The incidence of gall-bladder disease is increased two-fold amongst users of oral contraceptives and is two and a half times higher amongst post-menopausal women taking oestrogen (Boston Collaborative Drug Surveillance Program, 1973, 1974). Both cholesterol and bile salt concentrations are increased in bile with oral contraceptives (Davis and Freston, 1972). The oestrogen-induced increase in biliary cholesterol concentration and reduction in the pool size and synthesis rate of bile acids has been confirmed by Pertsemlidis *et al* (1973a). During pregnancy in the human, the bile acid concentrations in plasma are unchanged from the non-pregnant situation (Sjövall and Sjövall, 1966). However, a single report noted a decrease in biliary cholesterol and bile acid concentrations in the second half of pregnancy (Large *et al*, 1960).

Sterol balance studies have been carried out on 7 women at 3 stages during normal pregnancies in order to further elucidate possible mechanisms associated with the hyperlipaemia of pregnancy.

METHODS

A. EXPERIMENTAL DESIGN

Subjects. Sterol balance was studied during each of the three trimesters of pregnancy in 7 women. The group had a mean age of 26.9 years (range 25-29 years), and included both nulliparous and multiparous women. Table 3.1 gives the details of each subject. The study was conducted with subjects remaining in a free-living situation.

Diet. A diet of approximately constant cholesterol composition was designed for each woman for use in the study periods, with the aim of making it as similar to her usual diet as possible. Each woman was instructed in the weighing and preparation of the cholesterol-containing foods and the need for rigid adherence to the diet. The prescribed diet was commenced 10 days prior to a faecal collection period, and continued throughout the 8 days of collection.

Collections. It was decided not to use an inert faecal marker, although it seemed most unlikely that chromium sesquioxide would be teratogenic. Corrections for faecal flow could therefore not be made, but it was hoped that sterol excretion would be averaged in an 8 day collection of faeces. Faeces were collected directly into plastic bags, and deep frozen.

Fasting blood samples were collected at 4 to 5 week intervals during pregnancy, at least one lipid estimation being carried out during each study period.

B. LABORATORY METHODS

Plasma Lipids. Plasma cholesterol and triglyceride were estimated in a Technicon Auto Analyser Mark II Colorimeter. Plasma lipoproteins were separated by preparative ultracentrifugation and precipitation (Chapter 2).

Measurement of Faecal Neutral Sterols and Bile Acids. The method used was that of Miettinen *et al* (1965) and Grundy *et al* (1965).

Extraction The faeces were collected into two 4-day pools and each pool was homogenized with water. Duplicate aliquots of about 2 gm were taken for

TABLE 3.1

Women in the Sterol Balance Studies: Personal and Dietary Details

Name	Age (years)	Parity	First Trimester Gestation (weeks)	Weight (kg)	Plasma Cholesterol (mg/100 ml)	Plasma Triglyceride (mg/100 ml)	Dietary Cholesterol (mg)
N.B.	29	2	10	63.6	227	166	454
R.B.	27	0	11	55.8	226	103	443
R.C.	25	1	8	60.4	229	87	213
H.M.	27	1	9	45.4	182	86	420
R.S.	25	0	10	60.4	221	117	450
C.v.d.S.	28	1	10	63.6	149	53	426
C.W.	27	1	12	55.4	233	98	420

neutral sterol and bile acid extraction and analysis. Internal standards of 5-10,000 dpm of labelled cholesterol and bile acid were carried through the procedure. The aliquots were saponified with 2 mls of 10 N sodium hydroxide and 18 mls of 95% ethanol and refluxed for 1 hour over a steam bath. Ten mls of water were added to the cooled samples and the neutral sterols were extracted into petroleum ether (B.P. 60-80°C), (3x50 mls, 1x25 mls). The neutral sterols were separated into cholesterol, coprostanol and coprostanone bands by thin layer chromatography using a heptane:ether; 45:55 solvent system. Each band was eluted separately. An aliquot of the cholesterol band was taken for counting to calculate recovery, which averaged 90%. A known aliquot of 5-α cholestane was added to each band to act as internal standard during gas liquid chromatography.

The ethanolic solution containing the bile acids was evaporated almost to dryness. Following the addition of 5 mls of 2 N sodium hydroxide it was further saponified at high pressure (2 atmospheres) for $2\frac{1}{2}$ hours. After cooling to room temperature, the bile acids were extracted at pH 2 into chloroform:methanol; 2:1 (v:v) (60 ml) and chloroform (2x40 ml). The large volume was reduced to dryness in a rotary evaporator, and the extract redissolved in 10 ml of chloroform:methanol; 2:1. A one ml aliquot was taken for measurement of radioactivity to calculate bile acid recovery. The remaining 9 ml were evaporated to dryness, and the bile acids methylated at room temperature overnight, using 4 ml of 5% HCl in dry methanol. Following methylation, the mixture was evaporated to dryness and redissolved in 9 ml of chloroform:methanol; 2:1. The methylated bile acids were separated by thin layer chromatography of a 4 ml aliquot using first a benzene solvent to separate the fatty acid methyl esters. The plates were then run in a second solvent, iso-octane: isopropanol:acetic acid; 60:20:0.5. The bile acids were eluted with methanol, evaporated to dryness and redissolved in 4 ml of ethyl acetate,

from which 1 ml was taken to check for losses. To the remaining 3 ml, 130 µg of 5-α cholestane was added.

Gas Chromatographic Analysis The aliquots containing the sterols plus 5-α cholestane were evaporated to dryness. Fifty µl of silylating agent (Tri-Sil, Pierce Chemical Co., Illinois, U.S.A.) was added 30 minutes prior to injection. Standards of cholesterol and methyl cholate were similarly silylated and analyzed repeatedly. The gas chromatograph (Packard Series 7800) contained a 6 foot, coiled, glass column, packed with 1% DC-560, 100/120 mesh on Gas-Chrom Q (applied Science Lab., State Park, Pennsylvania, U.S.A.). The following conditions were operative:

Inlet temperature	260°C
Column temperature	240°C
Flame ionization detector temperature	250°C
Outlet temperature	250°C
Carrier gas (N_2) flow rate	60 ml/min
Hydrogen flow rate	40 ml/min
Air flow rate	375 ml/min
Column inlet pressure	28 psi

RESULTS

A. PLASMA LIPIDS

The plasma cholesterol and triglyceride concentrations during pregnancy are shown in Table 3.2. They are very similar to the changes previously described amongst the larger group of subjects (Chapter 2).

B. STEROL BALANCE DURING PREGNANCY

1. Bile Acid Excretion There was some variation between individuals which was of the order found in non-pregnant women. The mean bile acid

TABLE 3.2

Plasma Lipid Changes During Pregnancy

Gestation (weeks)	n	Plasma Lipid Concentration (mg/100 ml)	
		Cholesterol	Trigylceride
9-11	6	206 ± 13.4*	102 ± 15.5
12-14	6	211 ± 17.0	85 ± 8.7
15-18	6	225 ± 13.4	119 ± 19.0
19-22	7	262 ± 13.6	143 ± 22.2
23-26	5	282 ± 18.4	166 ± 15.3
27-30	4	295 ± 12.2	180 ± 8.9
31-34	7	300 ± 15.3	251 ± 40.4

*concentration : mean ± S.E.M.

excretion was 155 mg/day (2.75 ± 0.96 mg/kg/day) during the first trimester (Table 3.3). Due to inter-subject variation, the mean of 197 mg/day found in the second trimester was not statistically different. However, the lower value of 152 mg/day in the third trimester was significantly different from the excretion in the second trimester ($p < 0.01$). Therefore, there may be an increase in bile acid excretion during the second trimester of pregnancy which returns during the third trimester to first trimester values.

2. Neutral Sterol Excretion The amount of total neutral sterol excreted in the faeces also varied between individuals to the degree found in published studies in men and non-pregnant women. The neutral sterol excretion within the group remained unchanged throughout pregnancy, as did the net sterol excretion, which is the sum of bile acid and neutral sterol excreted minus the dietary cholesterol.

3. Plasma Lipids and Sterol Excretion During the second trimester there was a positive correlation of 0.761 ($p < 0.05$, $n=7$) between the plasma cholesterol concentration and bile acid excretion. From only 5 samples in the second trimester, a correlation of 0.739 (not significant) was found between VLDL-cholesterol and bile acid output, and of -0.859 between HDL-cholesterol and bile acid excretion ($p < 0.1$).

There was a similar relationship between total plasma triglyceride concentration and bile acid excretion ($r=0.865$, $p < 0.02$, $n=7$), which was probably linked to VLDL-triglyceride ($r=0.685$), though this was not significant with only 5 measurements.

4. The Effect of Weight The rate of increase in body weight was normal in all 7 women. The individual weights during each collection period are shown in Table 3.4. There was no relationship between the increase in weight during pregnancy and change in sterol excretion ($r=0.019$). However, there was a negative relationship between the increase in body weight and the increase in plasma cholesterol in the first half of

TABLE 3.3

Excretion of Bile Acids and Neutral Sterols During Pregnancy

Subject	Gestation (weeks)	Neutral Sterols (mg/day)	Bile Acids (mg/day)	Total Sterols (mg/day)	Dietary Cholesterol (mg/day)	Net Sterol Balance (mg/day)
First Trimester						
N.B.	10	842	192	1034	454	580
R.B.	13	599	172	771	440	331
R.C.	11	440	142	582	212	370
H.M.	10	478	179	657	420	237
R.S.	15	403	70	473	450	23
C.v.d.S.	12	692	132	824	426	398
C.W.	14	850	201	1051	420	631
Mean ± S.D.		615 ± 186	155 ± 45	770 ± 219	400 ± 85	368 ± 206
Second Trimester						
N.B.	23	672	296	968	454	514
R.B.	22	592	222	814	380	434
R.C.	22	286	253	539	212	327
H.M.	22	396	122	518	420	98
R.S.	26	981	247	1228	450	778
C.v.d.S.	22	874	66	939	426	513
C.W.	22	469	171	640	420	220
Mean ± S.D.		610 ± 255	197 ± 81	807 ± 259	395 ± 84	411 ± 222
Third Trimester						
N.B.	34	660	186	846	454	392
R.B.	31	827	175	1002	380	622
R.C.	32	417	155	572	212	360
H.M.	32	522	105	628	420	208
R.S.	34	716	187	903	450	453
C.v.d.S.	38	1074	122	1195	426	769
C.W.	32	475	134	609	420	189
Mean ± S.D.		670 ± 229	152 ± 33	822 ± 232	395 ± 84	427 ± 211

pregnancy (-0.744, $p \simeq 0.05$, n=7).

TABLE 3.4

Changes in Weight During Pregnancy:

The Relationship to Plasma Cholesterol Changes

in the First Half of Pregnancy

Subject	Weight (kg) during collection			Increase Between Collections 1 & 2	
	1	2	3	Weight (kg)	Plasma Cholesterol (mg/100 ml)
C.W.	55.4	58.6	66.3	3.2	33
C.v.d.S.	63.6	66.7	73.1	3.1	37
R.B.	55.8	60.4	63.6	4.6	40
R.C.	60.4	63.6	72.2	3.2	48
N.B.	63.6	66.3	69.0	2.7	74
R.S.	60.4	63.1	66.1	2.7	98
H.M.	45.4	46.7	50.9	1.3	103

DISCUSSION

Neutral sterol and total sterol excretion remained unchanged throughout pregnancy, whilst there may have been an overall increase in bile acid excretion during the second trimester, which then returned to first trimester values during the third trimester. During the second trimester, bile acid excretion was directly related to the plasma cholesterol and plasma triglyceride concentrations. Whilst the increase in weight during pregnancy had no apparent effect upon sterol excretion, during the first half of pregnancy the increase in weight bore an inverse relationship to the increase in plasma cholesterol.

Ideal sterol balance studies demand maintenance of a steady-state in the subject, particularly with regard to constant sterol ingestion, steady body weight and unchanging plasma lipid concentrations. The latter two criteria could not be strictly upheld in studies carried out during pregnancy. The average weight gain per week during pregnancy varies with the time of gestation; up to the 16-18th week weight is gained at about 0.36 kg/week, between the 19th to 28th weeks 0.45 kg/week and then until term, 0.36-0.41 kg/week (Thomson and Billewicz, 1957). For practical purposes then, during any one collection period, the weight of the women in this study could be considered stationary. Plasma lipids change markedly during pregnancy, but once again, the increment which generally occurred over one collection period was small. One exception to this was during the first trimester collection in subject R.S., when the plasma cholesterol concentration increased from 221 to 254 mg/100 ml, while the net sterol excretion was only 23 mg/day.

Plasma cholesterol concentration need not be related to the rate of cholesterol production or to the amount of cholesterol in the two exchangeable pools either in normal subjects or in those with established hyperlipoproteinaemia (Nestel *et al*, 1969). Endogenous cholesterol synthesis (Nestel *et al*, 1969) and bile acid excretion (Miettinen, 1973; Nestel and Hunter, 1974) increase with body weight. The weight accumulated during pregnancy includes increased deposition of body fat (Hytten and Leitch, 1972). However, the sterol balance studies do not show a direct relationship between body weight or increase in body weight and excretion of sterols or bile acids. A relationship between plasma lipids and weight was demonstrable with a negative correlation between the increase in body weight and plasma cholesterol in the period of the first to second trimester. This may be an indication of movement of cholesterol between body pools and plasma as a function of altered synthesis, since changes in body mass alter the flux of cholesterol between

plasma and tissues (Nestel *et al*, 1973). The positive correlation between plasma cholesterol, plasma triglyceride and bile acid excretion is in accord with an increase in lipid synthesis during the second trimester and catabolism of cholesterol as bile acid.

Studies in women taking oral contraceptives have shown that the biliary bile salt and cholesterol concentrations are increased (Davis and Freston, 1972). In conjunction with this, pool size and synthesis rate of cholic acid are decreased (Pertsemlidis *et al*, 1973a). The oral contraceptive studies may simulate early pregnancy. A single study by Large *et al* (1960), in which samples of bile were taken from an indwelling T-tube following cholecystectomy showed that the biliary cholesterol, bile acid and phospholipid concentrations were high during the first trimester of pregnancy and maximal at about 3 months of gestation. In this woman biliary lipid concentrations subsequently decreased and rose again during the first week of the puerpurium. When ethinyl oestradiol was administered to male rats, there was an increase in biliary cholesterol concentration but with decreasing bile flow (Davis *et al*, 1973, 1974); low doses increased the degradation of cholesterol while a high dose reduced degradation. If the effects of oestrogens on cholesterol metabolism vary with the dosage, then the variable blood levels during pregnancy may explain the increase in bile acid excretion in some subjects during mid-pregnancy with a decrease in others. A recent study of bile salt kinetics in the pregnant baboon (Dietrick *et al*, 1973) may not be directly comparable to the situation in the human, the baboon undergoing a fall in serum cholesterol during pregnancy (van Zyl, 1957). Percutaneous aspiration of gall bladder bile was used in 4 animals prior to and during pregnancy. In all animals pregnancy resulted in a decrease in the chenodeoxycholate, but not in the cholate, pool size. However, there was a marked decrease in the synthesis rate of both bile acids and an increase in their half-lives during pregnancy.

CHAPTER 4

PLASMA LIPID CHANGES IN THE NEONATE AND DURING INFANCY

INTRODUCTION

The human infant has a very low plasma cholesterol level at birth (Whyte and Yee, 1958; Sweeney *et al*, 1961; Mortimer, 1964; Glueck *et al*, 1971; Barnes *et al*, 1972; Darmady *et al*, 1972), and in this respect is no different from other mammals (Shope, 1929; Friedman and Byers, 1961; Carroll *et al*, 1973). The cholesterol concentration has been found to be independent of maternal regulation, samples taken from different socioeconomic and ethnic backgrounds being similar despite differences in the maternal levels (Whyte and Yee, 1958; Mendez *et al*, 1959; Glueck *et al*, 1971). However, infants of diabetic mothers may have a higher umbilical cord cholesterol level than infants born to normal mothers (Mortimer, 1964; Pantelakis *et al*, 1964a). The sex of infants does contribute to some degree, females having a higher cholesterol level at birth than males (Barnes *et al*, 1972; Darmady *et al*, 1972).

The average plasma triglyceride level at birth is also low (e.g. Sweeney *et al*, 1961; Brody and Carlson, 1962; Kwiterovich *et al*, 1973). It is subject to environmental influence such as foetomaternal stress during parturition (Tsang *et al*, 1974a).

The lipoproteins in cord plasma differ from those in the normal adult both in absolute concentrations and in distribution (Glueck *et al*, 1971, 1973; Wille and Phillips, 1971; Kwiterovich *et al*, 1973).

Plasma lipid changes following birth have been monitored, with cholesterol increasing approximately 2-fold by the end of the first week (Darmady *et al*, 1972) and more slowly thereafter during the first year (Darmady *et al*, 1972; Friedman and Goldberg, 1973a; Greten/Schettler, 1973). An increase in free fatty acid concentration is seen within 1-2 hours of birth, becoming maximal between 4-24 hours (Chen *et al*, 1965; Laron *et al*, 1967) and preceeding an increase in plasma triglyceride or VLDL concentration (Zee, 1968). The LDL concentration also rises on the first day, with HDL increasing about 25% during the first week (Abrams

and Freeman, 1969).

The plasma cholesterol concentration during infancy responds readily to dietary changes. Short term dietary studies using substituted vegetable oil-milk formulas, the addition of corn oil to diets, or the use of soya bean products all lead to a decrease in plasma cholesterol accompanying the ingestion of increased amounts of linoleic acid (Fomon and Bartels, 1960; Sweeney *et al*, 1961, 1962; György *et al*, 1963; Darmady *et al*, 1972; Tsang *et al*, 1974b).

The study of the incidence of hyperlipaemias within families has defined the mode of inheritance of some of the hyperlipoproteinaemias. Familial Type II hypercholesterolaemia (Type IIa) has an inheritance consistent with an autosomal dominant pattern (Fredrickson and Levy, 1972) as has combined hyperlipoproteinaemia (Type IIb) (Goldstein *et al*, 1973a). The recognition of the association of abnormalities of lipid metabolism and raised plasma lipids with arteriosclerotic disease (e.g. 2nd International Symposium on Arteriosclerosis, 1970) and an increasing belief that the high incidence of premature cardiovascular disease in Western Society need not be inevitable, has led to greater emphasis on early identification of individuals at risk.

Clinical identification of infants with hyperlipoproteinaemia has been largely limited to marked examples, such as primary or genetic disease among those homozygous for Type IIa, or Type I and secondary hyperlipoproteinaemia in children with congenital biliary atresia. However, clinical manifestations of other primary hyperlipoproteinaemias (e.g. heterozygous Type IIa) are rare, and therefore the diagnosis is rarely made without blood tests (Fredrickson and Breslow, 1973). Several studies have explored the possibility of diagnosing familial hyperlipidaemia randomly at birth (Glueck *et al*, 1971; Darmady *et al*, 1972; Greten/Schettler, 1973; Goldstein *et al*, 1973b), or more specifically in infants of parents known to be heterozygous for a Type II

hyperlipoproteinaemia (Kwiterovich *et al*, 1973; Goldstein *et al*, 1973b). Children found to be hypercholesterolaemic at birth have been followed up in order to evaluate the usefulness of neonatal lipid measurement. Glueck *et al* (1971) (Tsang *et al*, 1974b) have found the cord blood cholesterol to be a useful index of future hypercholesterolaemia, and a collaborative study in Germany reached a similar conclusion regarding genetic hypercholesterolaemia, especially when the cholesterol was measured in LDL rather than whole plasma (Greten/Schettler, 1973). However, Darmady *et al* (1972) in London concluded that genetic hypercholesterolaemia could not be reliably diagnosed on the basis of umbilical cord values. There was nevertheless a significant relationship between the cord cholesterol level and the concentration at 12-24 months (Darmady *et al*, 1972; Tsang *et al*, 1974b). Studies of umbilical cord measurements have been confined to the problem of hypercholesterolaemia since the other hyperlipoproteinaemias are rare in childhood (Fredrickson and Levy, 1972).

This chapter describes the follow-up of a large number of infants in whom plasma cholesterol and triglyceride concentrations were measured at birth.

METHODS

A. MEASUREMENT OF PLASMA LIPIDS IN SMALL BLOOD SAMPLES DURING INFANCY

Of two possible methods for collecting blood, the heel prick method was used rather than venepuncture. Following cleaning of the heel area, a small stab was made using a guarded lancette (Medipoint Inc., Mineola, N.Y., U.S.A.) and no further pressure was applied to minimize contamination with tissue fluid. The drops of blood which collected at the site were drawn into a heparinized capillary tube(s) (Chase Instruments Corp., Lindenhurst, N.Y., U.S.A., Cat. No. 501) by capillary action, until

60-100 µl had accumulated. Since the whole procedure was usually complete within the normal bleeding time, any further blood loss was stopped by release of constriction around the heel and direct application of pressure. One end of the microcapillary tube containing the blood sample was firmly sealed using Seal-ease (Clay-Adams Inc., N.Y., U.S.A., Cat. No. A-2980).

To separate the plasma, the sealed capillary tube was placed seal downwards into a protective plastic centrifuge tube, and spun in a bench centrifuge at 1,000 rpm for 2 minutes. The tube was scored with a glass file just above the buffy coat and carefully broken. The plasma was transferred directly by capillary action into a graduated 100 µl pipette. The known volume of plasma was then expelled directly into 2 mls of redistilled isopropanol. The cholesterol and triglyceride concentrations were analyzed in a Technicon Auto Analyser II Colorimeter (Manual, 1971).

B. VALIDATION OF THE METHOD

The cholesterol and triglyceride concentrations of 20 plasma samples were estimated using a small volume of plasma (20-50 µl) in 2 mls of isopropanol and compared with the result obtained from extraction of a large volume of plasma (0.5 ml) in 9.5 mls of isopropanol. The results are shown in Figures 4.1 and 4.2. The correlation coefficient for cholesterol concentration was 0.926 (Figure 4.1), with a regression equation of

$y = 7.5 + 0.93x$, where x and y are the large and small samples respectively.

The correlation coefficient for triglyceride concentrations from 15 samples was 0.933 (Figure 4.2), with a regression equation of

$y = 16.7 + 0.88x$.

Eight samples of plasma obtained by finger prick from adults were compared with those from venepuncture performed at the same time. There was no significant difference between the two groups of results, although the capillary method gave slightly lower values (Table 4.1).

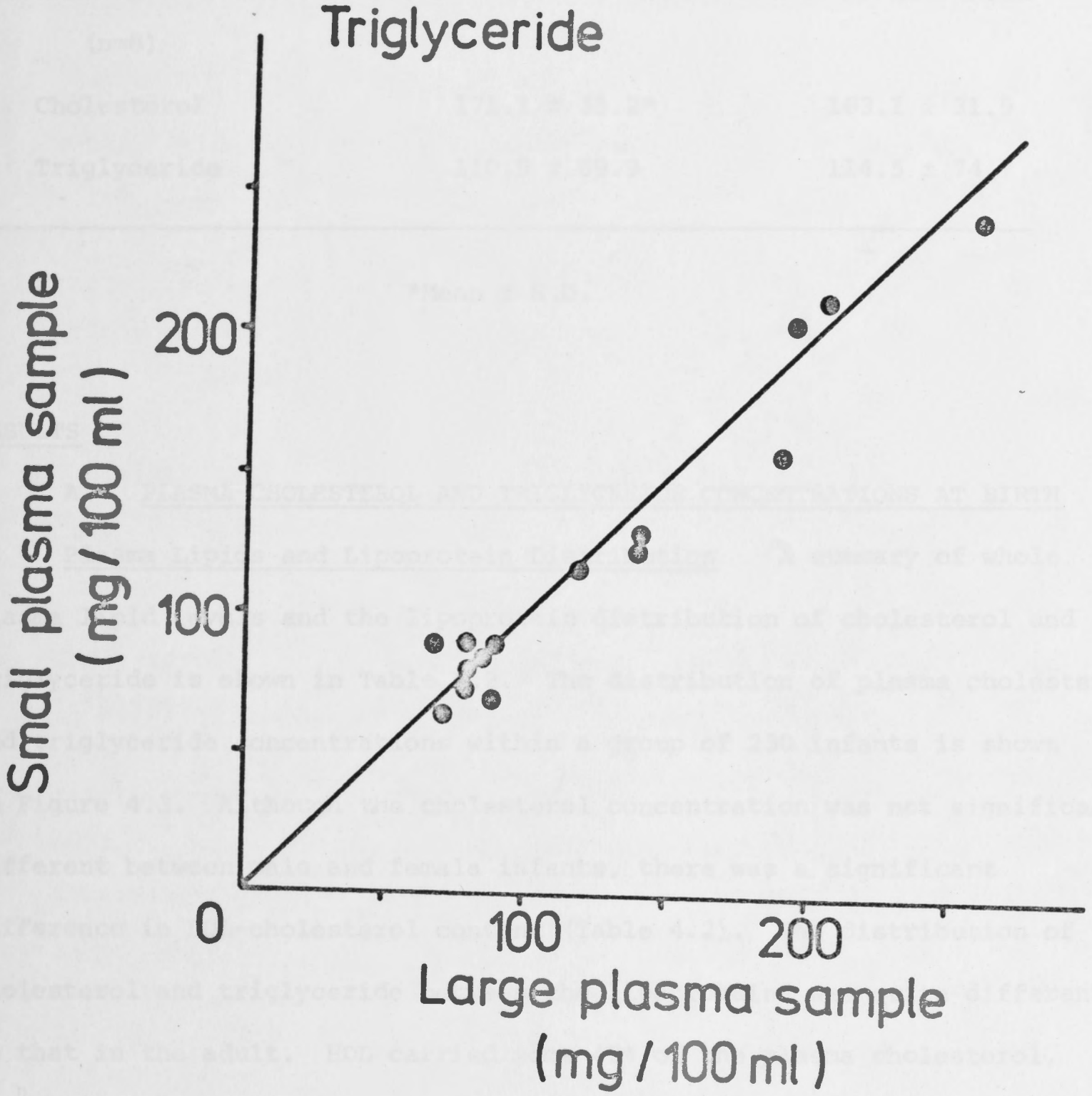


FIGURE 4.2 Comparison of Plasma Triglyceride Concentration Derived from Large and Small Plasma Volumes

The plasma triglyceride concentration of 14 samples was measured on a Technicon Auto Analyser II Colorimeter following extraction of 20-50 µl and 0.5 ml plasma in isopropanol as described in the text. The straight line represents the expected values if there is perfect agreement between the 2 extracts.

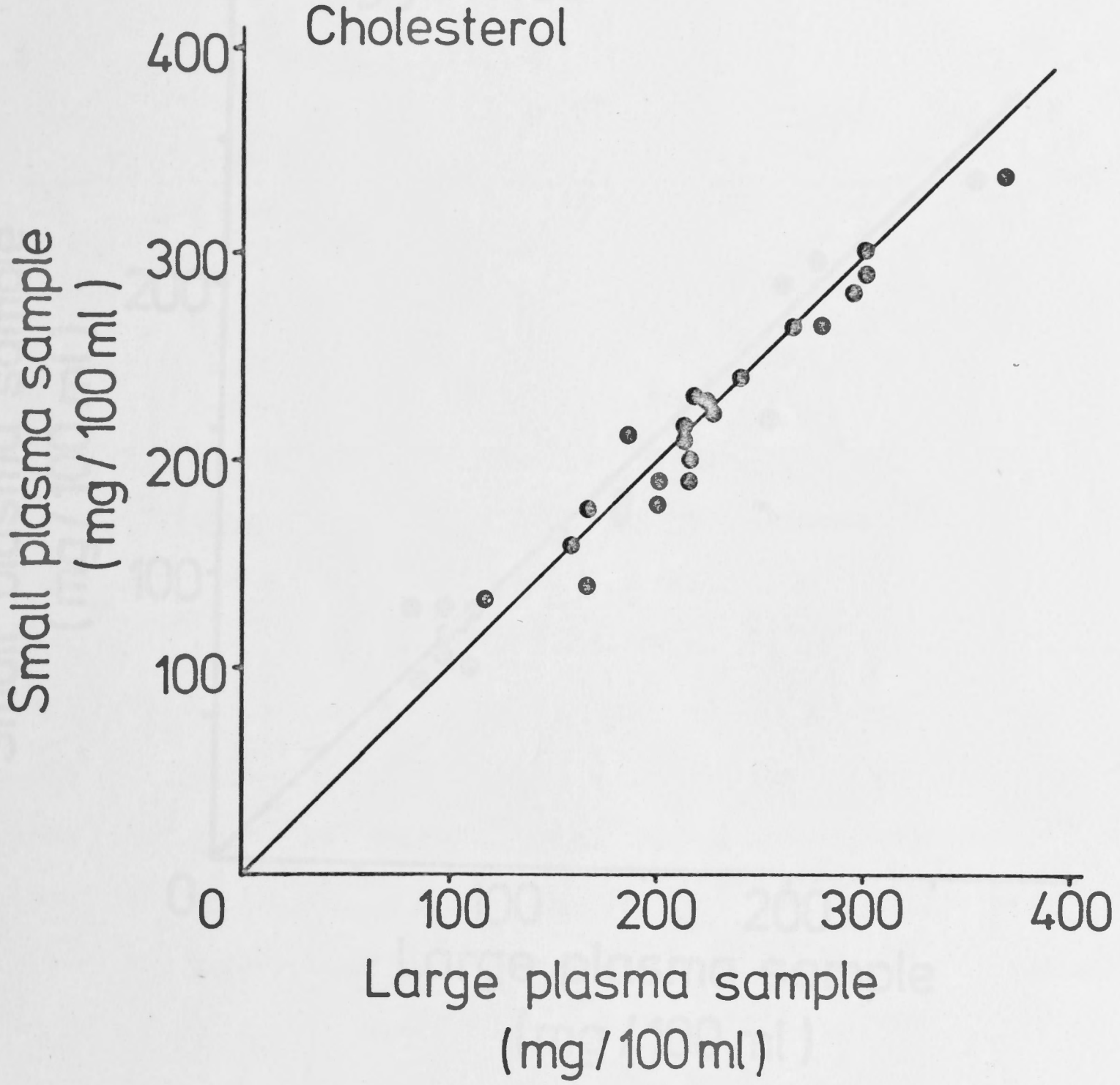


FIGURE 4.1 Comparison of Plasma Cholesterol Concentration Derived from Large and Small Plasma Volumes

The plasma cholesterol concentration of 20 samples was measured on a Technicon Auto Analyser II Colorimeter following extraction of 20-50 µl and 0.5 ml plasma in isopropanol as described in the text. The straight line represents the expected values if there is perfect agreement between the 2 extracts.

TABLE 4.1

Comparison of Plasma Analyses of Cholesterol Concentration

on Blood Obtained by Finger Prick and Venepuncture

Plasma Concentration (mg/100 ml)	Finger Prick (Small sample)	Venepuncture (Large sample)
(n=8)		
Cholesterol	171.1 ± 35.2*	183.1 ± 31.9
Triglyceride	110.9 ± 69.9	114.5 ± 74.7

*Mean ± S.D.

RESULTS

A. PLASMA CHOLESTEROL AND TRIGLYCERIDE CONCENTRATIONS AT BIRTH

1. Plasma Lipids and Lipoprotein Distribution A summary of whole plasma lipid levels and the lipoprotein distribution of cholesterol and triglyceride is shown in Table 4.2. The distribution of plasma cholesterol and triglyceride concentrations within a group of 230 infants is shown in Figure 4.3. Although the cholesterol concentration was not significantly different between male and female infants, there was a significant difference in LDL-cholesterol content (Table 4.2). The distribution of cholesterol and triglyceride between the lipoproteins was quite different to that in the adult. HDL carried some 45% of the plasma cholesterol, and only 50% was associated with LDL. The comparable adult distribution is 31% and 64% respectively. The major carrier of triglyceride in the newborn infant is LDL (47%), with 31% and 22% associated with VLDL and HDL respectively. The ratio of cholesterol to triglyceride was 0.29:1 in VLDL in the infant compared to 0.2:1 in the female adult, 1.47:1 in LDL in the infant versus 4.2:1 in the adult (1.76:1 in the delivering mother), and 2.7:1 in HDL in the infant and 3.1:1 in the adult. The

TABLE 4.2

Plasma Cholesterol and Triglyceride Concentrations

and Their Lipoprotein Distribution at Birth

Plasma Concentration (mg/100 ml)	No. of Infants	Total	VLDL	LDL	HDL
ALL INFANTS	29				
Cholesterol		94 ± 32*	6 ± 3	47 ± 18	42 ± 18
Triglyceride		68 ± 30	21 ± 10	32 ± 12	15 ± 7
MALES	12				
Cholesterol		86 ± 23	6 ± 2	39 ± 8	42 ± 18
Triglyceride		60 ± 15	20 ± 7	27 ± 10	13 ± 4
FEMALES	11				
Cholesterol		113 ± 40	6 ± 3	59 ± 24	48 ± 21
Triglyceride		79 ± 39	24 ± 15	37 ± 16	19 ± 10
NORMAL DELIVERIES	16				
Cholesterol		89 ± 23	6 ± 2	43 ± 14	41 ± 17
Triglyceride		57 ± 15	17 ± 6	28 ± 9	13 ± 4
COMPLICATED DELIVERIES	9				
Cholesterol		106 ± 46	6 ± 3	55 ± 26	45 ± 22
Triglyceride		81 ± 32	27 ± 10	37 ± 16	18 ± 10

Significant differences:

LDL-cholesterol:	Males vs females	p <0.02
Plasma triglyceride:	Normal vs complicated	p <0.02
VLDL-triglyceride:	Normal vs complicated	p <0.01
LDL-triglyceride:	Normal vs complicated	p >0.05
HDL-triglyceride:	Normal vs complicated	p >0.05

*Mean ± S.D.

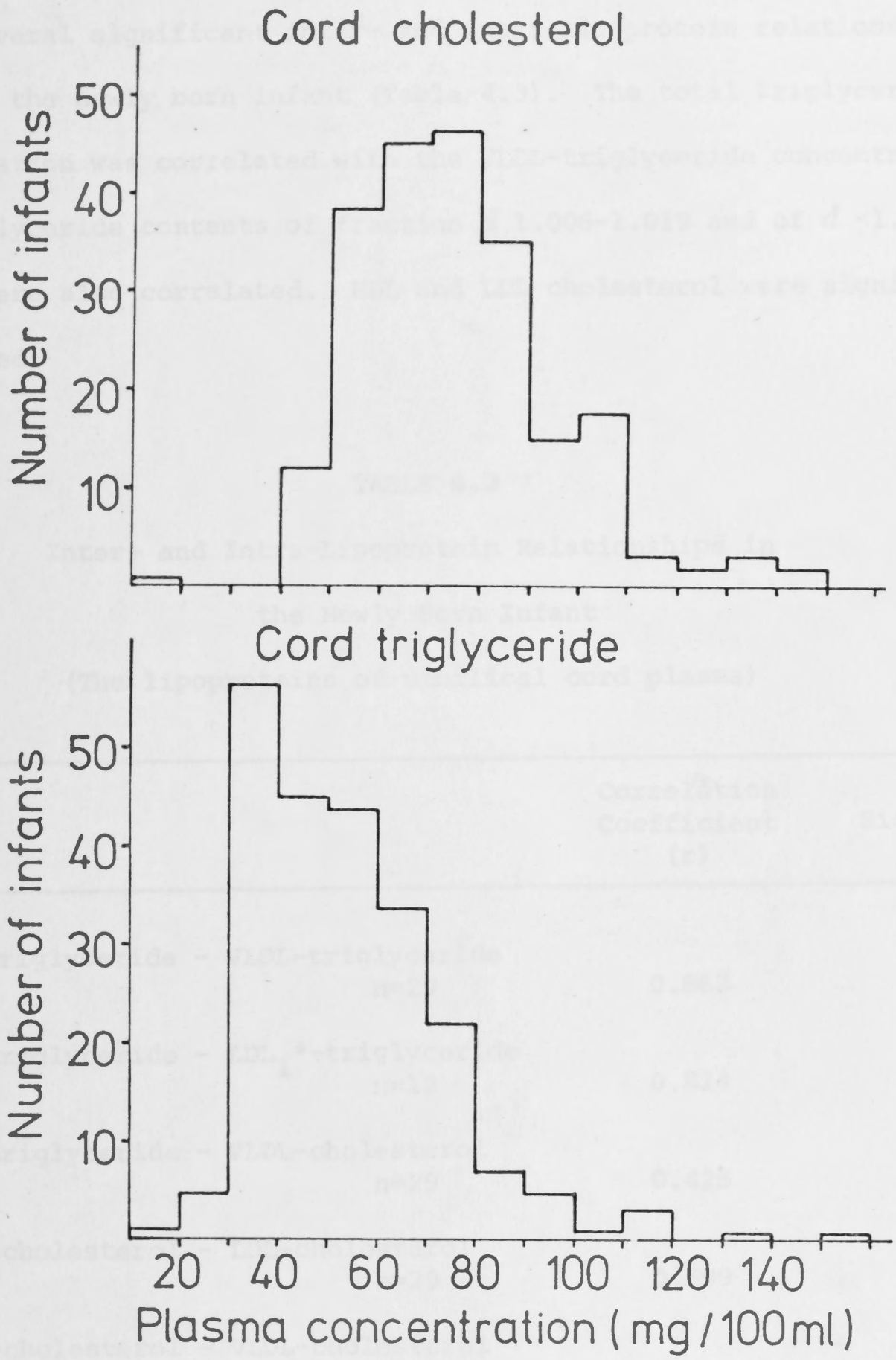


FIGURE 4.3 Distribution of Plasma Cholesterol and Triglyceride Concentrations in Umbilical Cord Plasma

The cholesterol and triglyceride concentrations were measured in the umbilical cord plasma collected from a group of 230 infants. They were a subgroup of 747 babies sampled in Canberra (Barnes *et al*, 1972), in whom plasma lipids were remeasured at 12-22 months of age.

distribution of lipids among the lipoproteins resembled that seen in pregnancy, though the absolute values were very much lower.

Several significant inter- and intra-lipoprotein relationships were found in the newly born infant (Table 4.3). The total triglyceride concentration was correlated with the VLDL-triglyceride concentration; the triglyceride contents of fraction d 1.006-1.019 and of d <1.006 (VLDL) were also correlated. HDL and LDL cholesterol were significantly correlated.

TABLE 4.3

Inter- and Intra-Lipoprotein Relationships in

the Newly Born Infant

(The lipoproteins of umbilical cord plasma)

	Correlation Coefficient (r)	Significance (p)
Plasma triglyceride - VLDL-triglyceride n=29	0.862	<0.001
VLDL-triglyceride - LDL_1*-triglyceride n=12	0.814	<0.01
VLDL-triglyceride - VLDL-cholesterol n=29	0.425	<0.05
HDL-cholesterol - LDL-cholesterol n=29	0.599	<0.001
HDL-cholesterol - VLDL-cholesterol n=29	-0.256	n.s.

*LDL_1 - lipoprotein density d 1.006-1.019

2. Maternal-Infant Lipid Relationships There was no significant correlation between maternal plasma cholesterol concentration at delivery and infant plasma cholesterol concentration (Table 4.4).

TABLE 4.4

Plasma Lipid Concentrations: Infant-Maternal Relationships

	Correlation Coefficient (r)	Significance (p)
AT DELIVERY		
Whole Plasma		
Maternal cholesterol - infant cholesterol n=122	0.188	n.s.
Maternal triglyceride - infant triglyceride n=122	0.243	<0.02
Lipoproteins		
Maternal LDL-cholesterol - infant cholesterol n=38	0.428	<0.01
Maternal HDL-cholesterol - infant cholesterol n=39	-0.100	n.s.
Maternal HDL-cholesterol - infant HDL-cholesterol n=17	0.497	<0.05
AT 6-7 WEEKS POST PARTUM		
Whole Plasma		
Maternal cholesterol (6 wks) - infant cholesterol (birth) n=78	0.159	n.s.
Maternal triglyceride (6 wks) - infant triglyceride (birth) n=79	0.031	n.s.
Lipoproteins		
Maternal LDL-cholesterol (6 wks) - infant cholesterol (birth) n=25	0.003	n.s.

However, among the individual lipoprotein fractions some relationships were demonstrable. Among a group of 38 infants, a significant positive correlation was found between the cholesterol concentrations in maternal LDL and in the infant whole plasma (Figure 4.4). A significant relationship was also found between the cholesterol concentration in maternal and infant HDL in a small group of 17.

There was a weak, but positive correlation between maternal and infant triglyceride levels. Since the maternal triglyceride concentration was increased in women suffering from pre-eclampsia, hypertension and renal disease, the effect of these conditions on the infant's lipids was examined separately as will be discussed later.

The maternal lipoproteins at the time of delivery reflect hormonal rather than genetic factors, so that a possible relationship between mother and child might be more realistically examined several weeks after delivery. This was attempted 6-7 weeks post partum, but as shown in Table 4.4, a significant relationship was not found.

3. Environmental Factors: The Effect on Umbilical Cord Triglyceride

The cord triglyceride concentration may partially reflect environmental factors operating on the mother and the foetus during parturition. The obstetric records and labour histories of 88 mothers and 89 babies (including one pair of twins) were examined retrospectively. Criteria regarding duration of labour and maternal hypertension are those of accepted obstetric practice. A summary of results is shown in Table 4.5.

(a) Maternal Factors The normal group consists of infants from normotensive labours, of normal duration and the result of vaginal deliveries, in whom no evidence of foetal distress was found. Hypertension is defined as blood pressure greater than 140/90 on more than one occasion during labour. Duration of delivery is normal if the first stage is less than 24 hours, and a normal second stage is less than 30 minutes. Foetal distress was diagnosed by a marked acceleration or

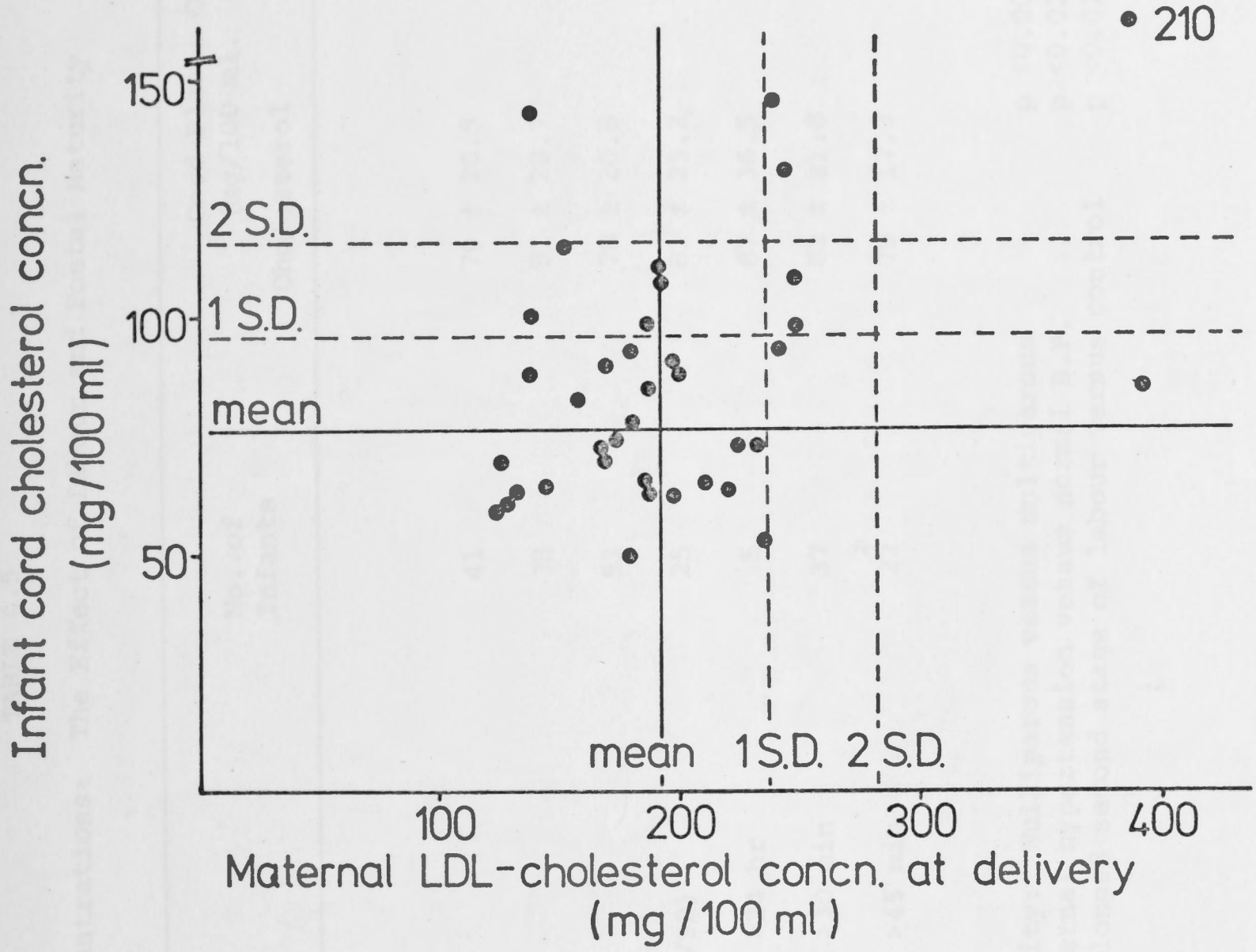


FIGURE 4.4 Relationship Between the Cholesterol Concentrations of Maternal LDL and Infant Whole Plasma

The LDL content of cholesterol in maternal plasma at delivery and in the babies whole plasma was measured in 38 pairs. The means and standard deviations of both groups are also shown.

TABLE 4.5

Plasma Lipid Concentrations: The Effect of Labour and Foetal Maturity

			No. of Infants	Cord Plasma Concentration (mg/100 ml. Mean ± S.D.) Cholesterol	Triglyceride
(a)	MATERNAL				
	No known complication (control)		41	79 ± 20.5	53 ± 14.0
	Parity: *Nulliparous*		38	81 ± 20.7	70 ± 20.8
	Multiparous		51	78 ± 20.6	56 ± 14.5
	Hypertension during labour (≥140/90)		25	81 ± 25.2	68 ± 20.0
	Prolonged labour: *First stage*	>24 hr	5	87 ± 36.5	63 ± 20.2
	Second stage	>30 min	37	82 ± 21.8	65 ± 19.8
		>45 min	22	79 ± 17.3	71 ± 21.3

Significant differences: (triglyceride)	Parity: Nulliparous versus multiparous	p <0.001
	Maternal hypertension versus normal B.P.	p <0.01
	Prolonged second stage of labour versus control	p <0.01

Continued

TABLE 4.5

(Continued)

	No. of Infants	Cord Plasma Concentration (mg/100 ml. Mean ± S.D.) Cholesterol	Triglyceride
(b) FOETAL			
No known complication (control)	30	78 ± 18.9	52 ± 13.4
Foetal distress	11	83 ± 24.1	69 ± 24.9
Low apgar (≤7)	21	81 ± 28.9	61 ± 16.4
Dysmaturity	3	86 ± 16.8	90 ± 9.5
Rhesus incompatability	6	73 ± 9.2	66 ± 22.5

Significant differences: (triglyceride)	Foetal distress versus control	p <0.01
	Low apgar versus control	p <0.05
	Dysmaturity versus control	p <0.001

deceleration of foetal heart rate.

In general, factors related to duration and severity of labour were reflected in the cord triglyceride level (Table 4.5). This was seen in nulliparous as compared to multiparous women, the former usually having longer labours and a higher incidence of pre-eclampsia. The occurrence of hypertension alone and prolongation of the second stage of labour also resulted in an elevation of cord triglyceride levels.

(b) Foetal Factors The normal group consisted of infants who were delivered vaginally and whose mothers had normal labours, the infants having Apgar scores of 9 and 10 at delivery. The Apgar score is a simple assessment of the infant's cardiovascular, respiratory and neuromuscular status following birth. In the group in whom foetal distress or an Apgar score of 7 or less was noted the cord triglyceride levels were statistically higher than normal. Dysmaturity was also associated with high cord triglyceride values. The small group of infants with Rhesus factor incompatibility did not have statistically abnormal triglyceride levels.

While these environmental factors did appear to influence the neonatal plasma triglyceride concentration, the cholesterol concentration was not affected.

B. CHANGES IN PLASMA CHOLESTEROL AND TRIGLYCERIDE DURING THE FIRST WEEK OF LIFE

The changes in plasma cholesterol and triglyceride over the first week of life were followed in 10 infants in whom cord lipid measurements were available. Blood samples were collected periodically during the first week. The mean values are shown in Table 4.6. All infants had been fed either glucose solution or diluted milk formula routinely in the nursery prior to the first sample. No attempt was made to fast the infants prior to sample collection; however, the last feed was always 3 hours previously. This contributed to the fluctuations in plasma

TABLE 4.6

Plasma Lipid Concentrations: Changes During the First Week of Life

	Age	Birth	0-24 hr	25-48 hr	49-72 hr	94-120 hr
Plasma concentration (mg/100 ml) (n)						
Cholesterol	mean	85	73	98	145	140
	S.D.	15.3 (10)	28.5 (6)	38.9 (4)	54.2 (6)	40.8 (9)
Triglyceride	mean	51	89	120	65	151
	S.D.	35.9	47.9	35.2	33.6	135.2
Weight (kg)	mean	3.638			3.445	
	S.D.	0.484			0.447	
Packed cell volume (%)	mean		54.4		50.5	
	S.D.		7.8		6.5	

Significant differences:	Cholesterol concentration:	Birth vs 49-72 hours	p <0.01
		Birth vs 94-120 hours	p <0.001
	Triglyceride concentration:	Birth vs 49-72 hours	p <0.05
		Birth vs 94-120 hours	p <0.01
	Weight:	Birth vs 49-72 hours	p <0.001
	Packed cell volume:	Birth vs 49-72 hours	p <0.001

triglyceride levels.

Plasma cholesterol concentrations were measured during the first 24 hours in 6 of the 10 infants, and in 5 out of 6 there was a decrease (Figure 4.5). In the remaining infant, a marked increase occurred within 8 hours of delivery, which in this infant was accompanied by a slight decrease in plasma triglyceride. In only one other infant was there a decrease in plasma triglyceride in the first 24 hours, and this occurred in a dysmature infant, whose plasma triglyceride at birth was 126 mg/100 ml.

Measurements made during the third day showed both plasma cholesterol and triglyceride to be generally higher than at birth (Table 4.6, Figure 4.5). Measurements carried out in 5 of the children on day 10 suggest that there may have been a slight reduction in plasma cholesterol and triglyceride, which still remained above the umbilical cord level.

The newborn infant is highly unstable in metabolic terms. Table 4.6 also shows the change in weight and packed cell volume during the first 3 days post partum. There is a strong negative correlation between the decrease in infant body weight during the first 3-4 days of life and the increase in plasma triglyceride which has occurred over the same period of time (expressing both as a percentage of the birth value): $r = -0.755$, $p < 0.05$ (Figure 4.6).

The type of milk fed to the infant, whether human milk or milk formula did not significantly affect the pattern of plasma lipid change during the first 10 days. This was also the case in the larger group of infants in whom measurements were made at birth and on day 5 (Table 4.7). There was a highly significant correlation between the cord plasma and day 5 plasma cholesterol concentrations ($r = 0.548$, $n=34$, $p < 0.01$).

C. PROSPECTIVE STUDY OF CHANGES IN PLASMA LIPIDS DURING INFANCY

1. Design During the months August 1971 to February 1972 blood samples were collected from all babies born at the Canberra Hospital.

FIGURE 4.5 Plasma Cholesterol Concentration: Changes During the First Week of Life

The plasma cholesterol concentrations of 10 infants in whom the cord cholesterol had been measured was sampled intermittantly during the first 7-10 days of life. The results are expressed in terms of a percentage change compared with the umbilical cord concentration (0%). All infants show an increase during the first 96-98 hours.

◒ Female infants

■ Male infants

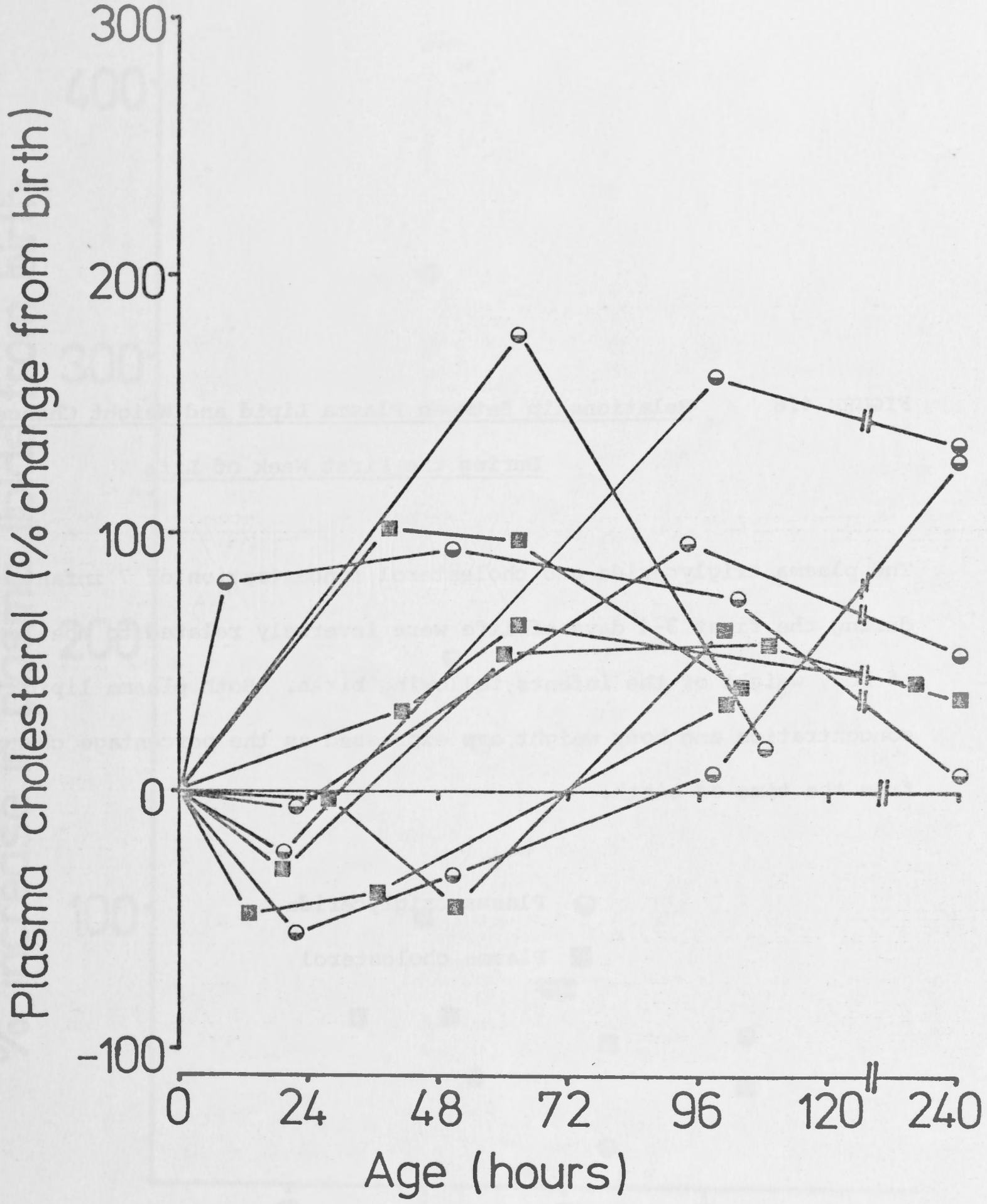

300
200
100
0
-100
Plasma cholesterol (% change from birth)
0
24
48
72
96
120
240
Age (hours)

FIGURE 4.6 Relationship Between Plasma Lipid and Weight Changes During the First Week of Life

The plasma triglyceride and cholesterol concentration of 7 infants during the first 3-4 days of life were inversely related to the loss of body weight of the infants following birth. Both plasma lipid concentration and body weight are expressed as the percentage change from the time of birth.

◓ Plasma triglyceride

▩ Plasma cholesterol

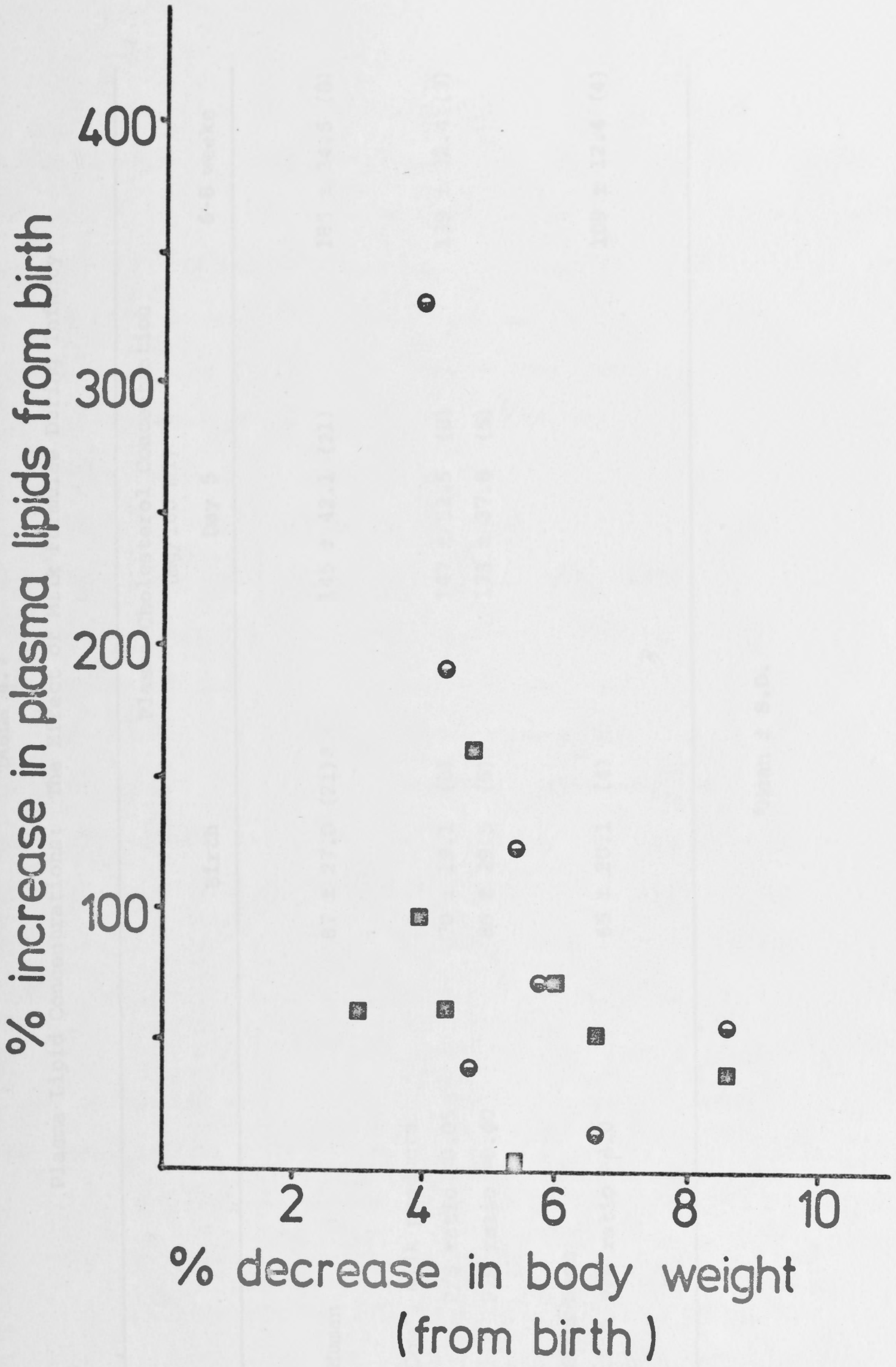

% increase in plasma lipids from birth
400
300
200
100
2
4
6
8
10
% decrease in body weight
(from birth)

TABLE 4.7

Plasma Lipid Concentrations: The Effect of Milk Formulae During Infancy

	Plasma Cholesterol Concentration (mg/100 ml)		
	Birth	Day 5	6-8 weeks
Human	87 ± 27.0 (21)*	145 ± 42.1 (21)	185 ± 34.5 (8)
Cow's milk products			
P/S ratio <0.05	70 ± 19.1 (8)	147 ± 52.5 (8)	139 ± 32.4 (3)
P/S ratio ≈0.40	86 ± 29.5 (5)	173 ± 37.8 (5)	
Soya bean			
P/S ratio >4.0	65 ± 20.1 (4)		109 ± 12.4 (4)

*Mean ± S.D.

The sample was collected at birth by the delivering obstetrician by venepuncture of the umbilical cord vein after the cord had been clamped prior to delivery of the placenta. The results obtained from 747 children have been previously reported (Barnes *et al*, 1972). Some of the children were retested at 12-22 months of age.

The parents of all children (with the exception of those who had been placed for adoption) were sent a brief letter, reminding them that a sample of blood had been collected at the time of birth and inviting them to have the child retested. Two hundred and thirty children were subsequently retested over a period of twelve months. Blood was collected early in the morning, with the child fasted for about 12 hours. The blood sample was collected from a heel prick as described earlier and the child was weighed. A short questionnaire regarding the infant's current dietary intake of milk and eggs and the family history of arteriosclerotic disease was completed by the mother.

The aims of the questionnaire were two fold: firstly to obtain information about the intake of cholesterol from milk and eggs and the approximate unsaturated fatty acid content of the diet, based on use of polyunsaturated margarine or oil and the use of milk infant formulae containing linoleic acid. The second aim was to gather information regarding the incidence of arteriosclerotic disease within the immediate family. Questions were directed towards the occurrence of heart attacks, strokes and angina and the age of occurrence, and the knowledge of hypercholesterolaemia or hypertension in the family. The immediate family was defined as including the infant's great-grand-parents and their children, i.e., a 4-generation span.

2. Plasma Cholesterol and Triglyceride Concentrations at 12-22 Months of Age The group was subdivided into those infants eating a highly saturated diet and those eating a more unsaturated diet. Several children were excluded because of intercurrent illness. The results from

the two groups are compared in the accompanying tables. Tables 4.8 and 4.9 show the mean plasma concentrations in both groups at birth and at the time of follow-up. Figure 4.7 shows the distribution of the plasma concentrations within the group. As can be seen, the plasma levels at 12-22 months are significantly lower in the group on the more unsaturated diet. There is no difference in the mean age, weight or dietary cholesterol intake between the two groups (Table 4.10). Subdividing the groups according to sex shows that the female infants generally have a lower level of cholesterol and triglyceride than the males during infancy, the reverse of the findings at birth. Table 4.8 also shows the groups subdivided according to blood groups. There is no apparent relationship between blood group and plasma cholesterol at birth or at the time of follow-up.

The correlation matrix (single correlation coefficients) for the group of infants eating the more saturated diet is shown in Table 4.11. Only three of the pairs had correlations which achieve significance, namely cord cholesterol and current plasma cholesterol ($r = 0.215$), current plasma triglyceride and plasma cholesterol ($r = 0.192$) and current weight and age (0.520).

The range of dietary cholesterol derived from milk and eggs was extremely wide, as demonstrated by the standard deviation in Table 4.10. The intake varied from virtually zero cholesterol from these products per week to over 3000 mg/week among a small group of children. The average consumption of milk was 20-22 ounces per day and eggs 2-3/day, irrespective of age. No estimate was made of additional intake from meat or other dairy products. No correlation was found between the dietary intake and the plasma cholesterol levels within the total population (Table 4.11). No difference in plasma levels could be found by taking the extreme ranges of the dietary intake of cholesterol (Table 4.12). However, among infants $\geq$18 months of age, the increase in plasma cholesterol from birth (expressed as a percentage of the birth level)

TABLE 4.8

Plasma Cholesterol Concentration at 12-22 Months:

The Effect of Diet, Sex and Blood Group

	No. of Infants	Plasma Cholesterol Concentration (mg/100 ml)	
		Birth	12-22 months
1. Saturated diet	175	77 ± 21.2*	194 ± 44.4
Males	86	74 ± 17.7	199 ± 48.8
Females	89	80 ± 23.8	189 ± 39.4
2. Unsaturated diet	26	79 ± 21.3	176 ± 40.6
Males	13	80 ± 24.9	181 ± 39.8
Females	13	77 ± 18.0	170 ± 42.1
3. Blood group			
Group O	76	77 ± 18.0	194 ± 42.1
Group A	66	76 ± 19.7	190 ± 43.8
Group B	12	74 ± 18.0	185 ± 35.6
4. Original sample (Barnes *et al*, 1972)			
All infants	747	76 ± 19.5	
Males	368	74 ± 18.2	
Females	379	79 ± 20.4	

Significant differences: 1. vs 2. All infants. $p < 0.05$
1. Males vs females - birth $p = 0.051$
4. Males vs females - birth $p < 0.001$

*Mean ± S.D.

TABLE 4.9

Plasma Triglyceride Concentration at 12-22 Months:

The Effect of Diet and Sex

		Plasma Triglyceride Concentration (mg/100 ml)		
	No.	Birth	No.	12-22 months
1. Saturated diet	180	56 ± 20.9*	138	101 ± 56.0
Males	82	53 ± 19.0	65	108 ± 61.0
Females	98	56 ± 20.4	73	94 ± 53.1
2. Unsaturated diet	26	55 ± 18.4	26	70 ± 40.6
Males	11	53 ± 18.7	11	74 ± 42.0
Females	13	57 ± 19.3	13	66 ± 40.9
3. Original sample (Barnes *et al*, 1972)				
All infants	747	52		
Males	368	52		
Females	379	52		

Significant differences: 1. vs 2. All infants. 12-22 mths. $p < 0.01$
1. vs 2. Males. 12-22 mths. $p = 0.08$
1. vs 2. Females. 12-22 mths. $p = 0.08$

*Mean ± S.D.

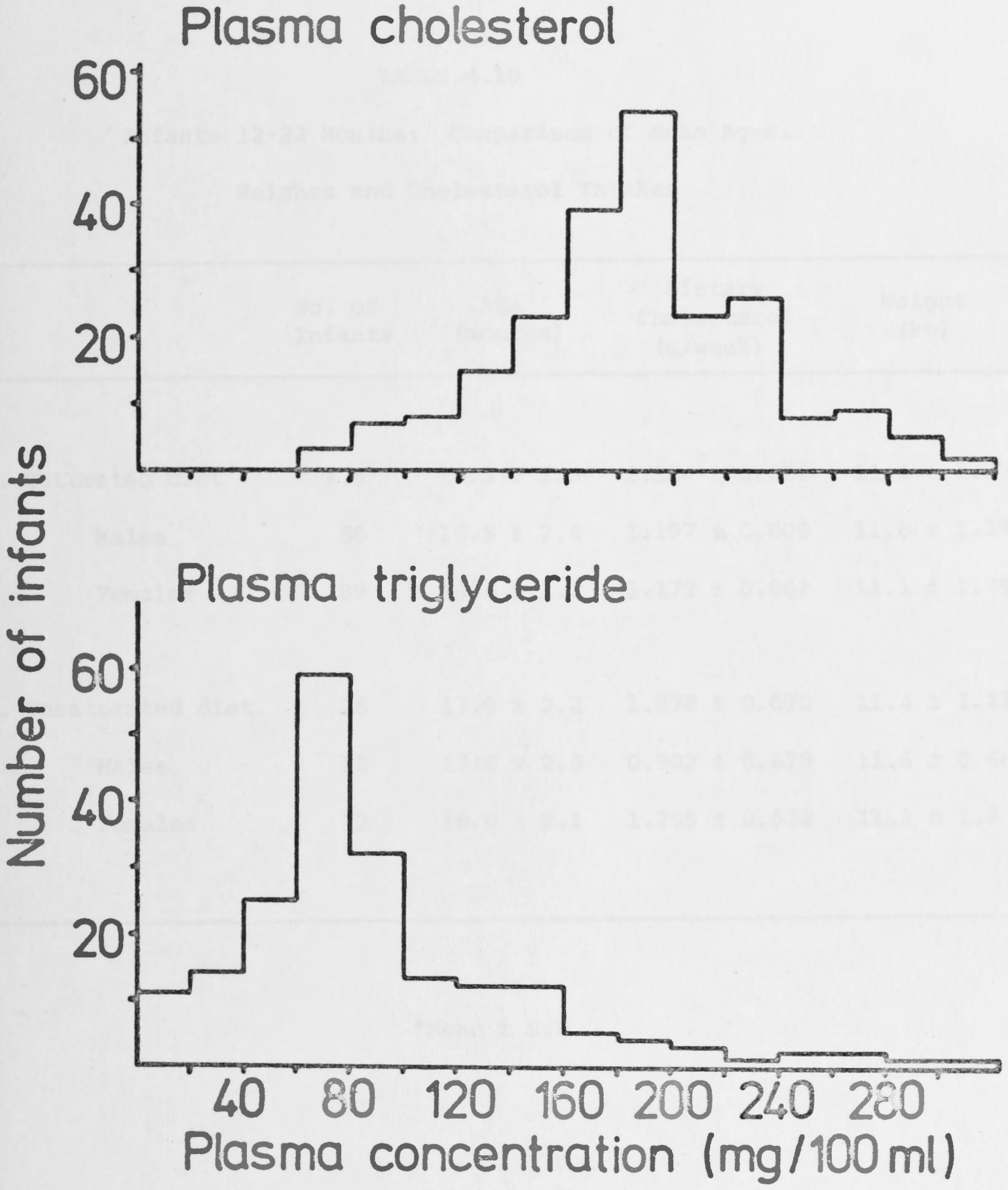


FIGURE 4.7 Distribution of Plasma Cholesterol and Triglyceride Concentrations at 12-22 Months of Age

Plasma cholesterol and triglyceride concentrations in plasma obtained by heel prick were measured in 230 infants between the ages of 12-22 months. These infants had previously been sampled at birth (Figure 4.3).

TABLE 4.10

Infants 12-22 Months: Comparison of Mean Ages, Weights and Cholesterol Intakes

	No. of Infants	Age (months)	Dietary Cholesterol (g/week)	Weight (kg)
1. Saturated diet	175	16.5 ± 2.6*	1.184 ± 0.635	11.4 ± 1.39
Males	86	16.5 ± 2.4	1.197 ± 0.609	11.8 ± 1.29
Females	89	16.4 ± 2.7	1.172 ± 0.662	11.1 ± 1.39
2. Unsaturated diet	26	17.9 ± 2.2	1.078 ± 0.670	11.4 ± 1.21
Males	13	17.8 ± 2.3	0.902 ± 0.678	11.6 ± 0.68
Females	13	18.0 ± 2.1	1.255 ± 0.638	11.3 ± 1.6

*Mean ± S.D.

TABLE 4.11

Plasma Lipid Concentrations at 12-22 Months: Correlation Matrix

	Cord Cholesterol	Birth Weight	Current Cholesterol	Current Weight	Age	Dietary Cholesterol	Current Triglyceride
Cord Cholesterol	1.000						
Birth Weight	0.077	1.000					
Current Cholesterol	0.215**	-0.056	1.000				
Current Weight	-0.178	0.175	0.049	1.000			
Age	-0.037	-0.005	-0.004	0.520***	1.000		
Dietary Cholesterol	-0.130	0.051	0.081	0.180	0.142	1.000	
Current Triglyceride	0.139	-0.009	0.192*	0.068	0.072	-0.046	1.000

n=174 (infants on saturated diet only)

*p ≃ 0.05

**p <0.05

***p <0.001

TABLE 4.12

Plasma Cholesterol Concentration at 12-22 Months:

The Effect of Dietary Cholesterol and Age

(a) Comparing low and high intake of cholesterol

	Dietary Cholesterol** from Milk and Eggs (<500 mg/wk)	(>2000 mg/wk)
Plasma cholesterol conc.	198 ± 49.3 (19)*	190 ± 48.0 (24)

*Mean ± S.D. (no.)

**Estimated from questionnaire

(b) Relationship between dietary cholesterol intake and plasma cholesterol expressed as percentage increase over the birth level

	No. of Infants	Correlation Coefficient (r)	Significance (p)
1. Saturated diet	175	0.169	<0.1
Age 12-14 months	61	0.145	n.s.
Age 15-17 months	42	-0.059	n.s.
Age ⩾18 months	72	0.309	<0.01
2. Unsaturated diet	25	-0.065	n.s.

correlated with dietary cholesterol intake (Table 4.12).

A positive correlation exists between body weight and plasma cholesterol concentration among the younger infants (aged 12-14 months) (Table 4.13) and between body weight and plasma triglyceride concentration among the older infants (aged ≥18 months). In the overall group, no relationship exists apart from age correlated with body weight (Table 4.11).

TABLE 4.13

Plasma Lipid Concentrations at 12-22 Months:

The Effect of Body Weight

	No. of Infants	Correlation Coefficient (r)	Significance (p)
1. PLASMA CHOLESTEROL			
Saturated diet	164	-0.052	n.s.
Aged 12-14 months	50	0.291	<0.05
Aged ≥15 months	113	0.024	n.s.
2. PLASMA TRIGLYCERIDE			
Saturated diet	124	0.068	n.s.
Aged ≤17 months	62	-0.025	n.s.
Aged ≥18 months	62	0.253	<0.05

Table 4.14 shows the distribution of infants according to their plasma cholesterol concentrations at birth and at 12-22 months. At birth, 7 children had plasma cholesterol concentrations greater than the mean plus 2 standard deviations (116 mg/100 ml) and 30 greater than 1

TABLE 4.14

Plasma Cholesterol Concentration at 12-22 Months:

Relationship to Umbilical Cord Cholesterol

Total 230/747*	Cord Plasma Cholesterol[1] Normocholesterolaemic (≤96 mg/100 ml) (<$\bar{x}$ + 1 S.D.)	Hypercholesterolaemic (>96 mg/100 ml) a. >$\bar{x}$ + 1 S.D.	Hypercholesterolaemic (>116 mg/100 ml) b. >$\bar{x}$ + 2 S.D.
Plasma Cholesterol Conc. at Age 12-22 mths.[2]	200	30	7
Normocholesterolaemic (<$\bar{x}$ + 1 S.D.) (≤238 mg/100 ml)	203	172	24	5
Hypercholesterolaemic (a) >$\bar{x}$ + 1 S.D. (>238 mg/100 ml)	27	21	6	2
(b) >$\bar{x}$ + 2 S.D. (>282 mg/100 ml)	7	7	0	0

*All infants, saturated and polyunsaturated diets

[1]Cord plasma: 76 ± 20 mg/100 ml (mean ± S.D.)

[2]Plasma cholesterol at 12-22 mths.: 194 ± 44 mg/100 ml (mean ± S.D.)

standard deviation. Of the former, at follow-up, 2 out of 7 infants had a cholesterol concentration greater than 1 standard deviation above the mean, while among the latter, 6 out of 30 showed a cholesterol level at follow-up that exceeded the mean by 1 standard deviation. Altogether 3 of these 6 mildly hypercholesterolaemic children at follow-up had a family history of hypercholesterolaemia. Of those infants that were normocholesterolaemic at birth, 21 (10.5%) were hypercholesterolaemic at follow up, 7 (3.5%) outside the 95th percentile. Therefore, many infants found to be hypercholesterolaemic at follow-up on the basis of the present statistical definition (greater than 1 standard deviation above the mean) were normocholesterolaemic at birth. The relative proportions of male and female children in the hypercholesterolaemic groups at birth and follow-up were different. At birth, among those with plasma cholesterol concentration greater than 96 mg/100 ml, the M:F ratio was 12:18; if greater than 116 mg/100 ml, the M:F ratio was 3:4. At follow-up, the ratios were reversed, with the M:F ratio being 17:10 at cholesterol levels above 238 mg/100 ml, and 4:3 at levels above 282 mg/100 ml. This means that a male baby who is hypercholesterolaemic at birth had a greater chance of remaining hypercholesterolaemic than a female infant.

3. The Relevance of Family History and Plasma Lipid Studies During Infancy The family history of each child has been analyzed for each of 3 events; the occurrence of either a heart attack or a stroke, which may or may not have resulted in death, before 50 years of age, the same occurrence in a family member older than 50 years, and the existence of a raised plasma cholesterol level in any member of the family. In a simple scoring system, a child could be positive for any one of these events only once, that is the history was either positive or negative for that classification.

The data for infants in whom plasma cholesterol and triglyceride concentrations at 12-22 months were available are shown in Table 4.15. The number of infants in each of the hyperlipidaemic groups is compared with the normolipidaemic infants, i.e. those whose cholesterol and triglyceride concentrations are less than 1 standard deviation above the mean. The number of children in whom familial hypercholesterolaemia has been identified is more than twice as high in the hyperlipidaemic groups as among the normolipidaemic infants. The number of early deaths in the hyperlipidaemic group is about 50% more than in the normal controls, whilst the prevalence of later death and disease is the same in all groups.

DISCUSSION

A. PLASMA LIPID CONCENTRATIONS AT BIRTH

1. Cholesterol The low plasma concentrations that were found at birth in Canberra were similar to those in many other countries; in the majority of studies the mean level lies between 60 and 79 mg/100 ml (e.g. Glueck *et al*, 1971; Darmady *et al*, 1972; Mortimer, 1964; Pantelakis *et al*, 1964a; Sweeney *et al*, 1961; Whyte and Yee, 1958; Zee, 1968). The 230 infants studied at follow-up did not vary significantly in their birth cholesterol and triglyceride concentrations from the larger population of 747 children from which they were drawn (Barnes *et al*, 1972). In that group, the maternal and infant plasma lipid concentrations were not significantly correlated. However, more detailed analysis of data in the smaller group showed several important interrelationships.

The relationship between whole plasma cholesterol concentrations in mother and infant was not significant ($r = 0.188$), in agreement with the findings in the larger population. However, study of the lipoprotein fractions was more fruitful. Among a subgroup of 38 maternal-infant

TABLE 4.15

Plasma Lipid Levels During Infancy: The Relationship to Family History

		A Normal Lipids	B ↑Cholesterol	C ↑Triglyceride	D Combined Hyperlipidaemia	B+C+D
Total Number of Infants		116	23	19	6	48
History of:	Early death or disease (<50 years)	17 (14.7%)	6 (26.1%)	4 (21.0%)	1 (16.7%)	11 (22.9%)
	Late death or disease (>50 years)	75 (64.6%)	17 (74.0%)	14 (73.6%)	3 (50.0%)	34 (70.8%)
	Hypercholesterolaemia	11 (9.5%)	5 (21.8%)	4 (21.0%)	3 (50.0%)	12 (25.0%)

A. Plasma cholesterol concentration <238 mg/100 ml
and Plasma triglyceride concentration <156 mg/100 ml
B. Plasma cholesterol concentration >238 mg/100 ml
and Plasma triglyceride concentration <156 mg/100 ml
C. Plasma triglyceride concentration >156 mg/100 ml
and Plasma cholesterol concentration <238 mg/100 ml
D. Plasma cholesterol concentration >238 mg/100 ml
and Plasma triglyceride concentration >156 mg/100 ml

pairs in whom the plasma lipoproteins had been separated, there was a positive correlation between the maternal LDL-cholesterol at delivery and the infant's whole plasma cholesterol. The cholesterol in HDL in the mother and the infant (n=17) were also correlated, possibly reflecting the intercorrelation between LDL and HDL cholesterol that was found in the infants.

Foetal tissues are capable of cholesterogenesis from early in gestation; this is particularly so in the liver where the *in vitro* and *in vivo* incorporation of acetate into cholesterol has been demonstrated (Popják and Beeckman, 1950; Chevallier, 1964; Givner and Jaffe, 1971). There is also *in vivo* transfer of cholesterol from the maternal circulation across the placenta (Chevallier, 1964; Connor and Lin, 1967; Hellig *et al*, 1970; Pitkin *et al*, 1972). In the human, the physical gradient is in favour of the foetus, but foetomaternal transfer probably also occurs to a small extent, as shown in the monkey (Pitkin *et al*, 1972). The proportion of cholesterol in the rat foetus which is derived from the maternal circulation is dependent upon the stage of gestation, decreasing with increasing maturity of the foetus (Chevallier, 1964). It has been estimated that at term 18-20% of the cholesterol in a human foetus is maternal in origin (Plotz *et al*, 1968; Hellig *et al*, 1970). If pregnant guinea-pigs are fed an hypercholesterolaemic diet during pregnancy, their foetuses have the same average plasma cholesterol concentrations at birth as those whose mothers did not receive cholesterol, but the proportion of foetal cholesterol derived from the mother is increased (Connor and Lin, 1967). The transfer of cholesterol across the placenta is increased under these conditions, resulting in a decrease in foetal cholesterogenesis. In the rabbit foetus, the plasma cholesterol is increased slightly if the mother is fed cholesterol (Popják, 1946), and the placenta shows marked morphological changes, with increased fat deposition on the foetal side (Popják, 1946; Zilversmit *et al*, 1972).

A positive correlation between maternal LDL-cholesterol at delivery and the infant plasma cholesterol has been found in the present study. This may reflect the known transfer of cholesterol across the placenta, although if this were the reason, one might expect the infant's cholesterol to rise even further. The other possibility, that of a genetic inter-relationship, may have been seen with maternal LDL rather than with whole plasma cholesterol, because the LDL concentration at delivery may more closely resemble the non-pregnant cholesterol level than does total cholesterol. It is also likely that the genetic expression of hypercholesterolaemia in the infant is better seen in the LDL-cholesterol rather than in the whole plasma cholesterol concentration (Greten/Schettler, 1973; Kwiterovich *et al*, 1973).

2. Triglyceride There was a weak positive relationship between the maternal plasma triglyceride concentration at delivery and the infant's cord plasma concentration ($r = 0.243$).

Maternal plasma glyceride does not cross the placenta to any significant extent (McBride and Korn, 1964). However, lipoprotein lipase-like activity has been demonstrated in the placenta *in vitro* (Mallov and Alousi, 1965).

The triglyceride concentration in the umbilical cord plasma was influenced by foetal and maternal factors. A similar observation has been made by Tsang *et al* (1974a). Maternal factors such as parity, the presence of hypertension, pre-eclampsia, prolongation of the second stage of labour, all resulted in an increase in the infant plasma triglyceride concentration. Foetal factors included dysmaturity, foetal distress and low Apgar scoring. Some of these factors were probably inter-related. For instance, the nulliparous woman tends to have a longer labour than the multiparous, and is prone to a greater incidence of pre-eclampsia. The foetus is more likely to suffer "foetal distress" when hypertension develops during labour, and a dysmature infant is particularly at risk.

As discussed previously (Chapter 2), the plasma triglyceride concentration is also higher in women who are hypertensive during the third trimester or at delivery.

The factor common to all these conditions is "stress", *in utero*, in the birth canal, or immediately post-natal. The plasma catecholamine concentration is raised in umbilical cord samples from infants born to mothers suffering from pre-eclampsia and in infants suffering from asphyxia neonatorum. The level of plasma pressor activity is significantly related to umbilical arterial P_aCO_2 and pH_a, and to the 1 minute and 5 minute Apgar scores (Holden *et al*, 1972). The catecholamines are derived from the foetus, there being little transfer from the maternal circulation (Adams *et al*, 1961). Catecholamine levels are 10 times higher in the dysmature infant with placental insufficiency than in a normal full term infant during the first 10-28 hours of life (Cheek *et al*, 1963).

Any relationship between high triglyceride levels and catecholamines is likely to be mediated through raised free fatty acid turnover. The plasma free fatty acid concentration of women at term is significantly higher than in non-pregnant women (Nelson *et al*, 1966; Becker *et al*, 1971; Fairweather, 1971) but is not demonstrably higher in women with pre-eclampsia than in women with normal pregnancies (Keele and Kay, 1966; Holden *et al*, 1972). There is transfer of free fatty acid across the placenta (van Duyne *et al*, 1962; McBride and Korn, 1964; Szabo *et al*, 1969) even against a small concentration gradient (van Duyne *et al*, 1962; Szabo *et al*, 1969). The rate of transfer and retention by the foetus is related to the maternal plasma concentration (Sabata *et al*, 1968). The low circulating levels of free fatty acid in the foetus have been offered as an indication of the low rate of transfer from the maternal circulation (van Duyne and Havel, 1959), but an alternative suggestion is of a high rate of utilization by the foetus (Nelson, 1965), since maternal free

fatty acid probably contributes to the laying down of foetal adipase tissue (Schwartz, 1968). Retention of free fatty acid by the foetus can be demonstrated by umbilical arterio-venous differences (Holden *et al*, 1972).

In vivo measurements of oxygen saturation during labour and at delivery indicate a marked relative hypoxia in the neonate during parturition, with saturations of only 50.8% (± 16.9, n=18) during the first stage of labour and 37.3% (± 14.2) during the second stage (mean ± S.D.) (Beard, 1974). Hypoxia stimulates catecholamine secretion (Greenberg *et al*, 1961; Cheek *et al*, 1963; Holden *et al*, 1972).

The plasma free fatty acid concentrations of infants born to mothers suffering from pre-eclampsia are lower than in normal infants at birth (Keele and Kay, 1966). This may be due to the elevated ketone concentration (Paterson *et al*, 1967), since infusions of ketones result in a depression of circulating free fatty acid in normal human adults (Fajans *et al*, 1964; Senior and Loridan, 1968) and children (Loridan and Senior, 1970; Binkiewicz *et al*, 1974).

In the presence of normal (Harris, 1974) or low (Keele and Kay, 1966) plasma free fatty acid concentrations in pre-eclamptic and dysmature infants respectively, the increased triglyceride concentration of the stressed neonate is not clearly related to free fatty acid metabolism, though the only available data is on free fatty acid concentration. This does not exclude an increased incorporation of free fatty acid into triglyceride, such as occurs in adults given ethanol, which also lowers plasma free fatty acid levels (Nestel and Hirsch, 1965).

B. PLASMA LIPID CHANGES DURING THE FIRST WEEK OF LIFE

1. Cholesterol During the first 24 hours there was a decrease in plasma concentration in 5 out of 6 of the infants, coupled with an increase in plasma triglyceride. In one infant in whom this early decrease in cholesterol was not observed, the plasma triglyceride decreased. The

plasma cholesterol level then increased in all infants during the next 3 days and then decreased slightly over the following week. At 6-8 weeks of age, breast-fed infants had significantly higher plasma cholesterol levels than infants fed on evaporated cow's milk.

Others have noted this increase in the plasma cholesterol towards the end of the first week (Kaplan and Lee, 1965; Darmady *et al*, 1972), both in LDL (during the first 24 hours) and in HDL (Abrams and Freeman, 1969; Studd *et al*, 1970). Close to term, the foetus is responsible for the synthesis of most of its cholesterol, though about 18-20% has probably been derived from the mother (Hellig *et al*, 1970). Following birth, any control exerted on foetal cholesterol metabolism by the mother or the placenta will be removed and synthesis of cholesterol might increase. In fact, cholesterogenesis in the neonate is depressed (e.g. Carroll, 1964; McNamara *et al*, 1972) and may reflect the operation of new controlling factors.

The suckling young of all species develop hypercholesterolaemia and significant increases in plasma cholesterol are seen during the first week (Carroll *et al*, 1973). Shope (1929) considered dietary cholesterol, particularly that in colostrum, to be responsible. Hypercholesterolaemia in calves is aggravated by cholesterol supplementation of milk (Wiggers *et al*, 1971). The high fat content of milk is also important, being itself sufficient to produce hypercholesterolaemia in the suckling rat (Harris *et al*, 1966). The combination of cholesterol and fat is necessary to produce hypercholesterolaemia in the suckling rabbit (Friedman and Byers, 1961) and calf (Carroll *et al*, 1973).

The incorporation of acetate (Carroll, 1964; Ballard and Hanson, 1967) and glucose (Ballard and Hanson, 1967) into cholesterol is depressed in the liver of the suckling rat, increasing to adult levels only upon weaning. The activity of HMG-CoA reductase declines rapidly in the post-natal period from the high value immediately prior to birth and

declines to very low levels before weaning (McNamara *et al*, 1972). Shah (1973) has shown that the incorporation of mevalonate into both non-saponifiable and digitonin-precipitable lipids was low in the suckling rat.

The question arises as to the nature of the inhibitor of HMG-CoA reductase activity during suckling. Incubation with either a supernatant fraction of a suckling rat liver homogenate or pre-incubation with rat milk decreases the activity of adult liver HMG-CoA reductase (McNamara *et al*, 1972). Cow and human milk have a similar inhibitory property demonstrated *in vivo* and *in vitro* (Boguslawski and Wróbel, 1974). Lastly of course, cholesterol itself is capable of feedback inhibition (Shah, 1973).

2. The Effect of Milk Composition in Later Infancy The infants who were being breast-fed at 6-8 weeks of age had higher plasma cholesterol levels than those infants on evaporated milk. Some studies have produced similar results (Fomon and Bartels, 1960; Darmady *et al*, 1972), while others have not (György *et al*, 1963; Woodruff *et al*, 1964). The linoleate content of human milk is higher than cow's (or evaporated) milk, and on this basis alone, the plasma cholesterol levels of children being breast-fed would be expected to be lower. However, among younger children, the absorption of fat is relatively inefficient (Fomon *et al*, 1970) and absorption of fat from cow's milk is less efficient than from human milk. This raises the possibility that any advantage which may be gained through higher linoleate content is outweighed by the greater absorption of fat.

3. Triglyceride The plasma triglyceride concentrations measured during the first week of life shows a general increase during the first 5 days, with a slight decrease over the next 4-5 days. Since measurements were made at the same time in relation to feeding, this fall may indicate an improvement in the clearance of triglyceride. The plasma triglyceride concentration at 48-72 hours of age was negatively related to the change

in body weight of the infant expressed as a percentage of the birth weight ($r = -0.755$, $p < 0.01$). This may reflect the utilization of fat stores for energy. In the normal infant, fat accounts for 16% of the total body weight at birth (Widdowson, 1950) as in the adult (Widdowson *et al*, 1951). It serves as the largest store of energy within the body (Dawes, 1968). Carbohydrate stores, concentrated as glycogen within the liver and skeletal and heart muscle (Shelley, 1964), are rapidly depleted following delivery. Liver carbohydrate falls to less than 10% of its birth level within 24 hours in a normal infant and within 12 hours in premature infants (Shelley and Neligan, 1966). There is therefore conversion from the utilization of carbohydrate *in utero* and at birth to that of fat. This leads to a rapid post-natal increase in plasma free fatty acid, glycerol and ketone concentrations, which is maximal at about 2 hours after birth (van Duyne and Havel, 1959; Chen *et al*, 1965; Keele and Kay, 1966; Harris, 1974). An inverse relationship between plasma free fatty acid and glucose is seen initially, the latter decreasing during the first 2 hours after delivery but then increasing, even in the presence of raised free fatty acid and the absence of food (Chen *et al*, 1965; Keele and Kay, 1966; Harris, 1974). The infant plasma triglyceride and VLDL concentrations increase significantly during the first 9 hours of life (Zee, 1968) probably secondarily to increased hepatic esterification of circulating free fatty acid (Havel, 1963).

C. PLASMA LIPIDS DURING INFANCY

1. Cholesterol The mean plasma cholesterol of the infants aged 12-22 months was 194 ± 44 mg/100 ml on the saturated diet, significantly higher than in the smaller group of infants who were eating a diet richer in polyunsaturated fatty acids (176 mg/100 ml). The male infants had higher plasma levels, but not significantly so. Blood groups were not a determinant of cord plasma cholesterol concentration, nor did they affect the cholesterol level during infancy, though it must be noted that much

larger samples might be required to demonstrate a relationship.

There was a significant correlation between the plasma cholesterol concentration in umbilical cord blood and the level during infancy among the children on the saturated fat diet ($r = 0.215$). Among the fewer children on the unsaturated fat diet the coefficient did not reach significance. At 5 days of age a highly significant relationship existed between cord and current plasma cholesterol concentrations ($r = 0.548$).

At the age of 12-22 months, there were two environmental factors, other than the fatty acid content of the diet, which showed important relationships with the plasma cholesterol concentration. The first was the infant's weight among the younger infants (12-14 months) and the second the dietary intake of cholesterol among the older infants ($\geq$18 months of age).

Estimates of the mean plasma cholesterol level among an infant population aged 12 months vary from 150 mg/100 ml (Friedman and Goldberg, 1973) to 191 ± 36 mg/100 ml (Darmady *et al*, 1972). Other results are biased by the choice of children according to their likelihood or otherwise of inheriting hypercholesterolaemia (e.g. Glueck and Tsang, 1972; Tsang *et al*, 1974). Neither of the 2 former studies (Friedman and Goldberg, 1973; Darmady *et al*, 1972) detected any significant difference in plasma cholesterol concentration between males and females, and neither study took any account of dietary factors.

In this current study, the fatty acid composition of the diet, whilst judged only on the use of milk formulae and polyunsaturated margarines and oils, had a significant impact on the plasma cholesterol levels during infancy. The cholesterol intake was approximately similar in the two groups of infants who were eating different kinds of fat. The response of the infant to the use of polyunsaturated fatty acids has been demonstrated many times, both close to birth (e.g. Sweeney *et al*, 1961; György *et al*, 1963; Darmady *et al*, 1972) and at several months of age

(Glueck and Tsang, 1972; Glueck *et al*, 1972; Tsang *et al*, 1974b).

Other surveys have also established a direct relationship between the umbilical cord cholesterol concentration and the plasma concentration during infancy. Darmady *et al* (1972) found a correlation coefficient of 0.27 (n=273) and Tsang *et al* (1974b) of 0.47, both for children on saturated and on unsaturated diets. No account was taken of the degree of saturation in the former study, even though the authors had previously shown differences in plasma cholesterol concentration according to the milk formula being used.

The relationship between the infants' weight and plasma cholesterol concentration was only demonstrable in this study among infants 12-14 months of age. The early increase in plasma cholesterol from birth to 6 weeks of age may be related to the infant's increase in weight (McKerrow, 1961). A significant correlation between the fat intake per day and the plasma cholesterol level is demonstrable in babies up to 4 months of age whether they are being breast-fed or formula-fed (McKerrow, 1962). Hames and Greenberg (1961) showed a correlation between plasma cholesterol and weight, which had been adjusted for height and age, amongst girls 6-11 years, but not amongst boys. Court *et al* (1974) in children 2-17 years and Hickie *et al* (1974) in males 11-18 years were unable to demonstrate any relationship between obesity (skin-fold thickness) and plasma cholesterol levels. In adults, body weight is related to cholesterol turnover, production being significantly correlated with excess weight (Nestel *et al*, 1969), but plasma cholesterol concentration and exchangeable cholesterol pool size are generally independent of turnover. Plasma cholesterol concentration has been directly, but weakly, correlated with body weight or fatness (Tanner, 1951; Gofman *et al*, 1952; Montoye *et al* (1966) but many studies have failed to demonstrate such a relationship (Lewis *et al*, 1957; Miller *et al*, 1958).

The epidemiological evidence implicating individuals with blood group A as being at high risk from thrombo-embolic and coronary heart disease (Nefzger *et al*, 1969; Kingsbury, 1971; Jick *et al*, 1969) has prompted other epidemiological investigations of risk factors within this group. Plasma cholesterol has been found to be higher among males having blood group A in some studies (Oliver *et al*, 1969; Langman, 1969). No difference in plasma cholesterol concentrations were found in either male or female infants at birth or at follow-up when they were examined according to their blood group. Saha and Banerjee (1971) also failed to find any difference at birth attributable to blood group.

2. Triglyceride The mean plasma triglyceride found in all infants was higher than anticipated, with wide standard deviations. Such variation is extremely suggestive of testing in the non-fasting state, particularly since the mean and standard deviation decreased with the age of the children. It may be that it is easier to fast an eighteen month infant than one of 12 months, and possibly the request not to feed the child may not have been sufficiently stressed. However, plasma triglyceride concentration was significantly lower among infants on the more polyunsaturated diet, and the females had significantly lower levels than the males. In infants 18 months and older, there was a positive but weak correlation relating body weight and plasma triglyceride concentration.

The plasma triglyceride concentration during childhood is 60-80 mg/100 ml among children 1-20 years (Hodges and Krehl, 1965; Glueck *et al*, 1973c; Kwiterovich *et al*, 1974; Court *et al*, 1974; Hickie *et al*, 1974; Wilmore and McNamara, 1974). Concentration is positively correlated with age amongst boys 11-18 years ($r = 0.22$, $n=613$) (Hickie *et al*, 1974). A relationship also exists between plasma triglyceride and subscapular skin-fold thickness (all children, including the obese, $r = 0.42$, aged 2-17 years [Court *et al*, 1974]; boys aged 11-18 years,

r = 0.18 [Hickie *et al*, 1974]). Among children diagnosed as having familial hypertriglyceridaemia approximately one-third are overweight (Glueck *et al*, 1973c). Weight reduction and a low fat diet result in a significant decrease of plasma triglyceride in the majority of children.

D. THE VALUE OF MEASURING PLASMA LIPID CONCENTRATIONS AT BIRTH AND AT 1-2 YEARS OF AGE

Of the original 747 babies sampled at birth, 230 infants were resampled at 12-22 months. For the purpose of analysis, hypercholesterolaemia was defined at birth as a cholesterol level greater than either 1 or 2 standard deviations (S.D.) above the mean (96 and 116 mg/100 ml respectively). The number of infants so classified was 30 and 7 respectively. At 12-22 months, hypercholesterolaemia was defined as greater than 1 S.D. above the mean (238 mg/100 ml). Out of 30 such infants, only 6 had had cord cholesterol levels greater than 96 mg/100 ml at birth and 2 greater than 116 mg/100 ml. The yield in terms of hypercholesterolaemia at 12-22 months was therefore 6 out of 30 (20%) and 2 out of 7 (28.5%) respectively (in terms of hypercholesterolaemia diagnosed at birth). The majority of children with hypercholesterolaemia at follow-up were normocholesterolaemic at birth.

Table 4.16 summarises the results from several trials which have been conducted to evaluate the efficacy of cord cholesterol measurements as an index of inheritence of hypercholesterolaemia. The majority have been designed to follow only those children defined as hypercholesterolaemic at birth and have not studied the fate of the normocholesterolaemic children.

In the study published by Darmady *et al* (1972), 274 out of 302 children were re-examined. Hypercholesterolaemia at birth was defined as 100 mg/100 ml (mean ± 1 S.D. = 78 ± 23 mg/100 ml) and 34 children fell into this category. Of these, 30 were retested as 12 months of age, at which time the mean plasma cholesterol was 191 mg/100 ml (S.D. = 36 mg/100 ml). Hypercholesterolaemia, arbitrarily defined as being greater

TABLE 4.16

The Predictive Value of the Diagnosis of Hypercholesterolaemia

Study/Author	No. of Infants	Umbilical Cord Cholesterol				
		Cholesterol (mg/100 ml)		No. of Infants		
		Mean	S.D.	Normal	$>\bar{x}$ + 1 S.D.	$>\bar{x}$ + 2 S.D.
American:[1]						
Glueck *et al* (1971)	1800	64	19			65
Tsang *et al* (1974b)						
German:[1,2]						
Greten *et al* (1973)	1323	60	20			92
Schettler (1973)		35 (LDL)	13			
				81		
English:[2]						
Darmady *et al* (1972)	302	78	23		34	
				268		
Kwiterovich *et al* (1973)[1]	29	100	15			16
		73	13	13		
Goldstein *et al* (1973b)[3]	2000					71 (Chol.)
						125 (Chol. and T.G.)
				134		
Canberra (1972-73)[2]	230 (747)	76	19			7/40
					30	

[1] LDL-cholesterol

[2] Whole Plasma

[3] Whole plasma cholesterol and triglyceride

Based on Umbilical Cord Plasma Measurements

Infants with Family History	Follow-up Cholesterol				
	Cholesterol (mg/100 ml)		No. of Infants		
	Mean	S.D.	$>\bar{x}$ + 1 S.D.	$>\bar{x}$ + 2 S.D.	$>\bar{x}$ + 1 S.D. with Family History
$^{17}/_{56}$ ($^{8}/_{56}$: 3 generation)	..... Dietary dependent				
$^{10}/_{73}$	113 (LDL)	33	$^{9}/_{61}$	$^{4}/_{61}$	$^{9}/_{9}$
$^{3}/_{81}$			$^{1}/_{65}$	$^{1}/_{65}$	$^{1}/_{3}$
?	191	36	$^{5}/_{30}$		$^{0}/_{5}$ $^{1}/_{274}$
$^{16}/_{16}$	>170 (LDL)			$^{11}/_{12}$	$^{11}/_{12}$
$^{13}/_{13}$	<170 (LDL)		$^{0}/_{7}$		
$^{37}/_{125}$ ($^{9}/_{125}$: 3 generation) $^{20}/_{134}$					
$^{3}/_{7}$ $^{6}/_{30}$	194	44	$^{2}/_{7}$ $^{6}/_{30}$		$^{2}/_{3}$ $^{3}/_{6}$

than 240 mg/100 ml a point mid-way between the first and second standard deviations, was found at follow-up in 24. However, only 5 of the 24 had also been hypercholesterolaemic at birth, i.e. 5 of the 30 initially hypercholesterolaemic infants. Family studies on all 5 were negative, and the only infant found to have familial hypercholesterolaemia was normocholesterolaemic at birth.

In the German study (Greten/Schettler, 1973) of 1323 unselected births a diagnosis of hypercholesterolaemia was made on the basis of both plasma cholesterol and LDL-cholesterol concentrations. A total of 92 infants had either plasma or LDL levels (or both) greater than 2 S.D. above the mean. Both parents were tested in 73 of these and in 10 cases type II hypercholesterolaemia was found. A control group of 81 normocholesterolaemic infants was randomly selected for follow-up, and among these, 3 parents were found to be hypercholesterolaemic. At 12 months of age, 61 initially hypercholesterolaemic and 65 initially normocholesterolaemic infants were resampled. LDL-cholesterol was used to define hypercholesterolaemia (greater than 1 S.D. being probable and greater than 2 S.D. definite type II hypercholesterolaemia). Where both the infant and a parent had been diagnosed as having hypercholesterolaemia (10 cases), 4 out of 10 infants had definite and a further 5 out of 10 probable hypercholesterolaemia at follow-up. In the control group, the corresponding proportion was 1 out of 3 (Table 4.16).

The large American series (Glueck *et al*, 1971; Tsang *et al*, 1974b) also provides follow-up data. The mean cord cholesterol level from 1800 unselected, consecutive births was 64 ± 19 mg/100 ml. The definition of hypercholesterolaemia was greater than 100 mg/100 ml and 65 infants were so diagnosed. Of the 65, 56 were followed for 12 months: one of the parents was found to be hypercholesterolaemic in 17 of the 56 (30.3%), a figure very similar to that reported by Goldstein *et al* (1973b) (Table 4.16). A diagnosis of familial hypercholesterolaemia was made in 8 of the

children, on the evidence of 3 generation vertical transmission and/or tendinous xanthomatosis. A control group was selected from the original 1800 infants, (normocholesterolaemic at birth and neither parent hyperlipidaemic). Both normocholesterolaemic and hypercholesterolaemic infants participated in dietary studies involving changes in dietary P/S ratio and cholesterol content (Glueck *et al*, 1972; Tsang *et al*, 1974b). At follow-up, the mean plasma cholesterol values on a low P/S ratio, high cholesterol diet were: normocholesterolaemic children with normolipidaemic parents, 135 mg/100 ml; hypercholesterolaemic children with normal parents, 164 ± 17 mg/100 ml (n=30); hypercholesterolaemic children of probable familial origin, 233 ± 59 mg/100 ml (n=12). On a diet with a high P/S ratio and low in cholesterol, this latter group of infants had a plasma level of 161 ± 27 mg/100 ml, i.e., the diet disguised the genetic trait. The normocholesterolaemic children had a mean of 131 mg/100 ml. The study also showed that children who are hypercholesterolaemic at birth and who have a family history of hypercholesterolaemia are most likely to remain hypercholesterolaemic during infancy.

A further study which emphasized the significance of genetic transmission in determining persistent hypercholesterolaemia is that of Kwiterovich *et al* (1973). Twenty nine children of a known hypercholesterolaemic parent were examined at birth and 19 were retested between the ages of 1-2^{1}/2 years. Classifying the children on the basis of LDL-cholesterol levels, 16 (55%) fell outside the 95th percentile at birth; 12 of the 19 were retested, and 11 of these 12 remained hypercholesterolaemic. The twelfth child had been placed on a cholesterol-lowering diet and was normocholesterolaemic. Similarly, the infants who were normocholesterolaemic at birth remained so at follow-up (7/7) despite having a hypercholesterolaemic parent.

Goldstein *et al* (1973b) studied 2000 consecutive births and determined the incidence of hyperlipoproteinaemia in the families (parents and grandparents) of 134 infants born with normal plasma lipids and of 125 in whom plasma triglyceride and/or cholesterol were outside the 95th percentile. Evidence of 3 generation transmission was obtained in 9 of the 125 hyperlipidaemic infants; in 5 the defect was familial hypercholesterolaemia; in 4, combined hyperlipidaemia (CHL). This gave a minimal estimate of heterozygote frequency for hyperlipidaemia of 9/2000 or 0.45%, 0.25% for hypercholesterolaemia and 0.20% for CHL. This compares with 1/274 or 0.37% (Darmady *et al*, 1972), 8/1800 or 0.44% (Tsang *et al*, 1974b) and 10/1323 or 0.75% (Greten/Schettler, 1973) for hypercholesterolaemia. Goldstein and his colleagues found that among the babes with high cord lipids 37 (26.4%) had one parent who was hyperlipidaemic, compared with 20 (14.9%) in the normolipidaemic group. A similar figure was found in this current study regarding the prevalence of probable familial hyperlipidaemia.

Infants who have been found to be normocholesterolaemic at birth have received little attention, and yet it appears that these children will form the majority of future hyperlipidaemic subjects. For instance, in this current study 24 of the hypercholesterolaemic infants at 12 months had been normocholesterolaemic at birth (80%). A similar figure was derived by Darmady *et al* (1972): 19 out of 24 or 79%. However, it would seem that this group of infants may have a higher percentage of families with known hypercholesterolaemia (20.8%) and a history of early arteriosclerotic disease (33.3%) compared to the normolipidaemic infants (9.5% and 14.7% respectively).

It has been shown that the cord plasma triglyceride may be related as much to environmental as to genetic factors. The plasma concentrations at 12-22 months were higher than anticipated and higher than levels generally quoted for children 1-20 years of age (Section C.2). Taking this into consideration, a diagnosis of hypertriglyceridaemia was made

if the child's plasma triglyceride, assumed to be fasting, was greater than 150 mg/100 ml (> mean ± 1 S.D.). Among these infants the family history contained a reference to hypercholesterolaemia or early arteriosclerotic disease more often than among the normocholesterolaemic infants.

There have been some studies in children concerned with the diagnosis of familial hypertriglyceridaemia in childhood, either as Type IV or combined hyperlipidaemia (CHL). Among 77 children born into families where one parent had CHL, only 39% were found to have hyperlipidaemia (Glueck *et al*, 1973d). Twenty three of these had a raised cholesterol (6 with raised triglyceride), whilst only 9 had an elevation of triglyceride alone. In a study of familial hypertriglyceridaemia (Type IV), 113 children, 1-20 years, from 36 propositi, were examined by Glueck *et al* (1973c): only 20% were found to be hypertriglyceridaemic, though among offspring older than 21 years of age, 10 out of 17 (58%) were involved. During childhood there seems to be incomplete penetrance of the hypertriglyceridaemic trait. That age may alter the penetrance of an hyperlipidaemic gene was also concluded by Kwiterovich *et al* (1974) in a study of 236 children resulting from 90 matings of parents with Type II hyperlipoproteinaemia. Between the ages of 1-19 years, 45% of the children were involved, but if this were further subdivided according to age, the prevalence was 52% if younger than 10 years and only 39% between 10 and 19 years.

In this current study being reported, the infants who were hypercholesterolaemic (n=23), hypertriglyceridaemic (n=19) or both (n=6) at 12-22 months had family histories of higher prevalence of early arteriosclerotic disease and a greater likelihood of a family history of hypercholesterolaemia. Goldstein *et al* (1973b) found the overall prevalence of measured lipid abnormality and death from coronary heart disease to be the same in the relatives of the normolipidaemic and hyperlipidaemic infants defined at birth.

In summary it would appear that infants found to be hypercholesterolaemic at birth are more likely than normocholesterolaemic infants to be hyperlipidaemic at 12-22 months, and to come from families with a history of lipid abnormality, which may be expressed as early arteriosclerotic disease. Nevertheless, the majority of infants who are hypercholesterolaemic or hypertriglyceridaemic at 12-22 months were normocholesterolaemic at birth, though they appear to show a higher prevalence of familial involvement than infants who remain normolipidaemic. Environmental factors such as dietary cholesterol, the saturation of fat and infant weight are important determinants of hyperlipidaemia and may disguise the true incidence of familial hyperlipidaemia.

CHAPTER 5

STEROL EXCRETION IN THE NEONATE AND YOUNG INFANT: THE INFLUENCE OF AGE AND DIET

INTRODUCTION

The human infant, like all mammalian young, is born with a low plasma cholesterol level, which rapidly increases over the first week of life (Pomeranze *et al*, 1958; Kaplan and Lee, 1965; Darmady *et al*, 1972). The "hypercholesterolaemia of suckling" regresses upon weaning in several animal species (Carroll *et al*, 1973), but not in the human infant. Several studies in children of European origin have shown a slow steady increase in plasma cholesterol levels through childhood and adolescence to values found in the adult population (Whyte and Yee, 1958; Hames and Greenberg, 1961; Godfrey *et al*, 1972; Friedman and Goldberg, 1973a).

The origin of foetal cholesterol is two-fold, from *de novo* synthesis within the foeto-placental unit and from the maternal circulation. The human foetus is capable of cholesterogenesis from early in gestation, the highest rate of synthesis being within the liver (Solomon *et al*, 1967; Telegdy *et al*, 1970a; Mathur *et al*, 1970; Givner and Jaffe, 1971). The placenta, which is an important site for the synthesis of steroid hormone derived from cholesterol (Talegdy *et al*, 1970b) is capable of synthesizing only minor amounts of cholesterol itself (van Leusden and Villee, 1965; Zelewski and Villee, 1966). The relative amount of cholesterol derived from the maternal circulation varies according to the age of the foetus, decreasing with maturity in the rat (Chevallier, 1964), and according to the species of animal (Chevallier, 1964; Connor and Lin, 1967; Pitkin *et al*, 1972). It seems likely that 18-20% of the cholesterol in a human infant may be derived from the maternal circulation (Hellig *et al*, 1970).

The same basic pathway of cholesterol synthesis probably operates in the foetus as in the adult, with intermediates such as squalene and lanosterol having been identified (van Leusden *et al*, 1971). However, additional pathways may operate in the placenta (Zelewski and Villee, 1966). It is known that in the rat HMG-CoA reductase is the major rate controlling enzyme of cholesterol synthesis in the foetus at term and in the

suckling animal (Harris *et al*, 1967; McNamara *et al*, 1972). Other rate controlling points have been sought, Shah (1973) demonstrating low rates of incorporation of mevalonate into non-saponifiable lipids and digitonin-precipitable sterols in the suckling, but not in the weanling rat. 7-dehydrocholesterol reductase activity is low in the liver of the foetal and newborn rat, reflecting the low rates of sterol synthesis, but increases rapidly during weaning (Wróbel, 1972). However, since 7-dehydrocholesterol does not accumulate, the reductase is unlikely to be a rate-limiting enzyme.

Bile acids are present in the gall bladder (Bongiovanni, 1965), meconium (Sharp *et al*, 1971; Back and Ross, 1973) and duodenum (Poley *et al*, 1964) of the human neonate. Any secondary bile acid present in the infant at birth is due to placental transfer from the maternal circulation (Lester *et al*, 1972). As well as cholic, chenodeoxycholic, lithocholic, ursocholic and deoxycholic acids being present in human meconium, 3β-hydroxy-5-cholenoic acid has been found in premature and mature neonates, in decreasing quantities with increasing maturity (Back and Ross, 1973). Two pathways for bile acid synthesis may exist side by side in the foetal liver. The first is the sequence operating in the adult liver, the rate limiting enzyme being cholesterol-7α-hydroxylase (Danielsson *et al*, 1967; Shefer *et al*, 1970), resulting in cholic and chenodeoxycholic acids. The alternative pathway is via 3β-hydroxy-5-cholenoic acid, lithocholic acid and chenodeoxycholic acid, involving cholesterol-26-hydroxylase in the first step (Mitropoulos and Myant, 1967). The foetal dog has been used as a model to investigate the enterohepatic circulation of bile acids. Near term, the liver will take up, conjugate and secrete tracer doses of cholic acid (Jackson *et al*, 1971) with reabsorption of bile acid in the intestinal tract (Smallwood *et al*, 1972).

Cholesterol is the precursor of bile acids, the latter serving also as the major route of excretion of cholesterol. In the adult, disruption

of bile acid metabolism affects cholesterogenesis directly, without the complicating factor of altered cholesterol absorption (Hamprecht *et al*, 1971). In the suckling rat, surgical severance of the bile duct leads to a 2-3 fold increase in cholesterol synthesis within 24 hours (Harris *et al*, 1967) with an increase in HMG-CoA reductase activity (McNamara *et al*, 1972). Cholic acid infusion immediately suppresses this increase in cholesterol synthesis (Harris *et al*, 1967). In the foetal dog, biliary drainage results in an increase in the conversion of cholesterol to bile acid (Lester *et al*, 1972).

Cholesterol turnover reflects the balance between the input of cholesterol from endogenous synthesis in tissues and from dietary cholesterol and the loss of cholesterol, which occurs mainly in the faeces in the form of cholesterol, bile acids and their degradation products (Nestel, 1970). Cholesterol turnover may be described in terms of a two-pool model (Nestel *et al*, 1969); pool A, the more rapidly turning over the the two pools, probably consists of the plasma, erythrocytes and viscera such as the liver and intestine, whilst pool B relates to adipose, skin and skeletal tissues (Chobanian and Hollander, 1962). Ideal studies of sterol balance demand steady state conditions including constant sterol intake, constant body weight and steady plasma cholesterol concentration. Cholesterol absorption increases proportionately to the increase in dietary cholesterol (Connor and Lin, 1974). Cholesterol and the fatty acid composition of the diet are both important determinants of plasma cholesterol concentration (Mattson *et al*, 1972; Keys *et al*, 1965a,b). However, plasma cholesterol concentration is not related to the synthetic rate of cholesterol, but rather to the fractional clearance from pool A (Nestel *et al*, 1969). In studies lasting 3-4 weeks, polyunsaturated fatty acids have been shown to increase the catabolism and excretion of cholesterol in adults (Moore *et al*, 1968; Nestel *et al*, 1973).

The aim of this study was to measure cholesterol and bile acid synthesis in the human neonate and during infancy, and in particular the response to dietary changes in fatty acid composition and cholesterol content.

METHODS

The aims of the study were 2-fold. The first was to measure the excretion of cholesterol, bile acids and their degradation products periodically during infancy. The second was to determine the effect of cholesterol-free soy milk and cholesterol containing cow's milk on cholesterol metabolism during infancy.

A. EXPERIMENTAL DESIGN

Four infants were studied till up to 5 months of age. All infants were carried through a normal pregnancy and delivery. They were being bottle-fed from birth by choice. A summary of the experimental design with the relationship in time between diet and sample collections is shown in Table 5.1. Infants were weighed regularly from the time of birth. Their weights are shown in Figure 5.1. The individual details of infants are given in Table 5.2.

Three infants (J.C,, S.F. and A.S.) were studied from birth to 56 days of age. Studies were commenced prior to the discharge of mother and baby from hospital on the sixth day post-partum. The infants were fed an evaporated cow's milk mixture for the first 25 days, then changed to a soy bean (cholesterol free) milk until 40 days of age. For the final 16 days they reverted to the initial cholesterol-containing evaporated milk. Collections of faeces were made on days 5-10, 19-25, 35-40 and 50-56 inclusive.

A fourth infant (L.K.) was maintained on the soy milk for two months from 25 days of age. At this stage cholesterol, in the form of egg yolk

TABLE 5.1

Experimental Design of Diet and Sample Collections During Sterol Balance Studies in Infancy

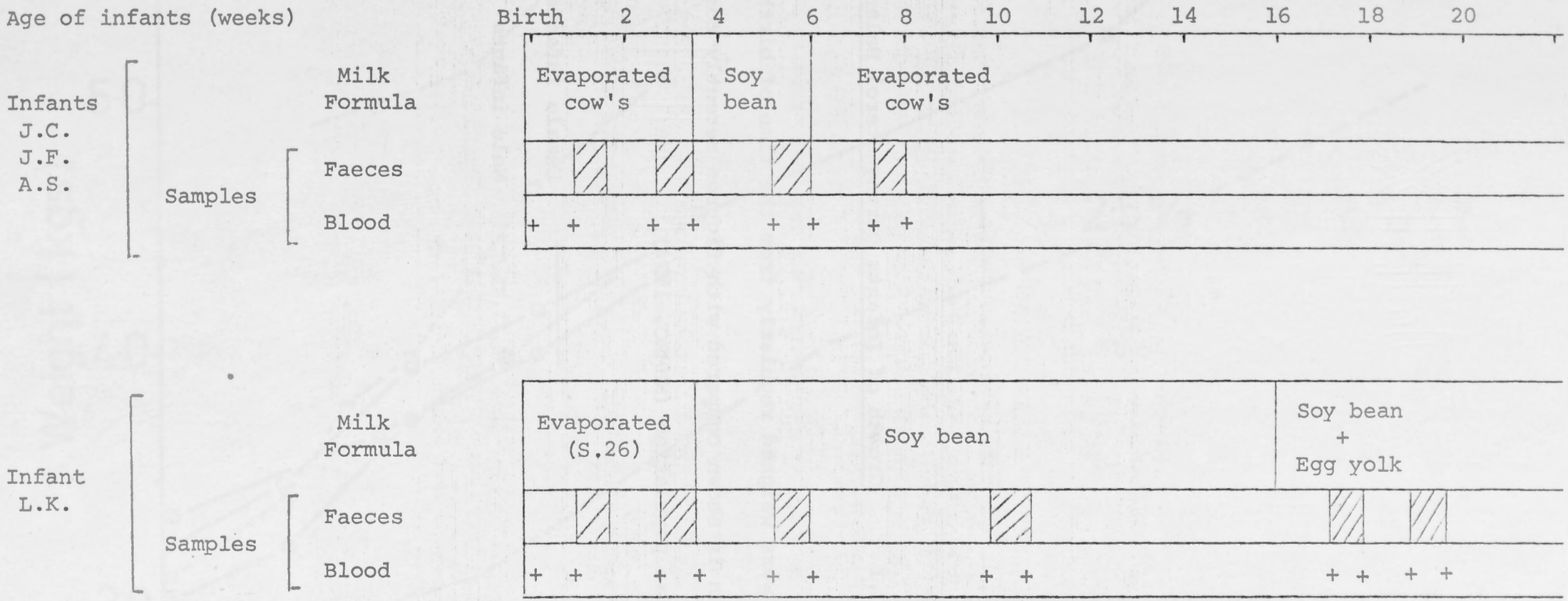


FIGURE 5.1 Growth of Infants During Sterol Balance Studies

Infants were weighed regularly from the time of birth. Their rate of growth is shown compared with figures recently compiled for an Australian population (NHMRC, 1974).

———— Female infants

– – – – Male infants

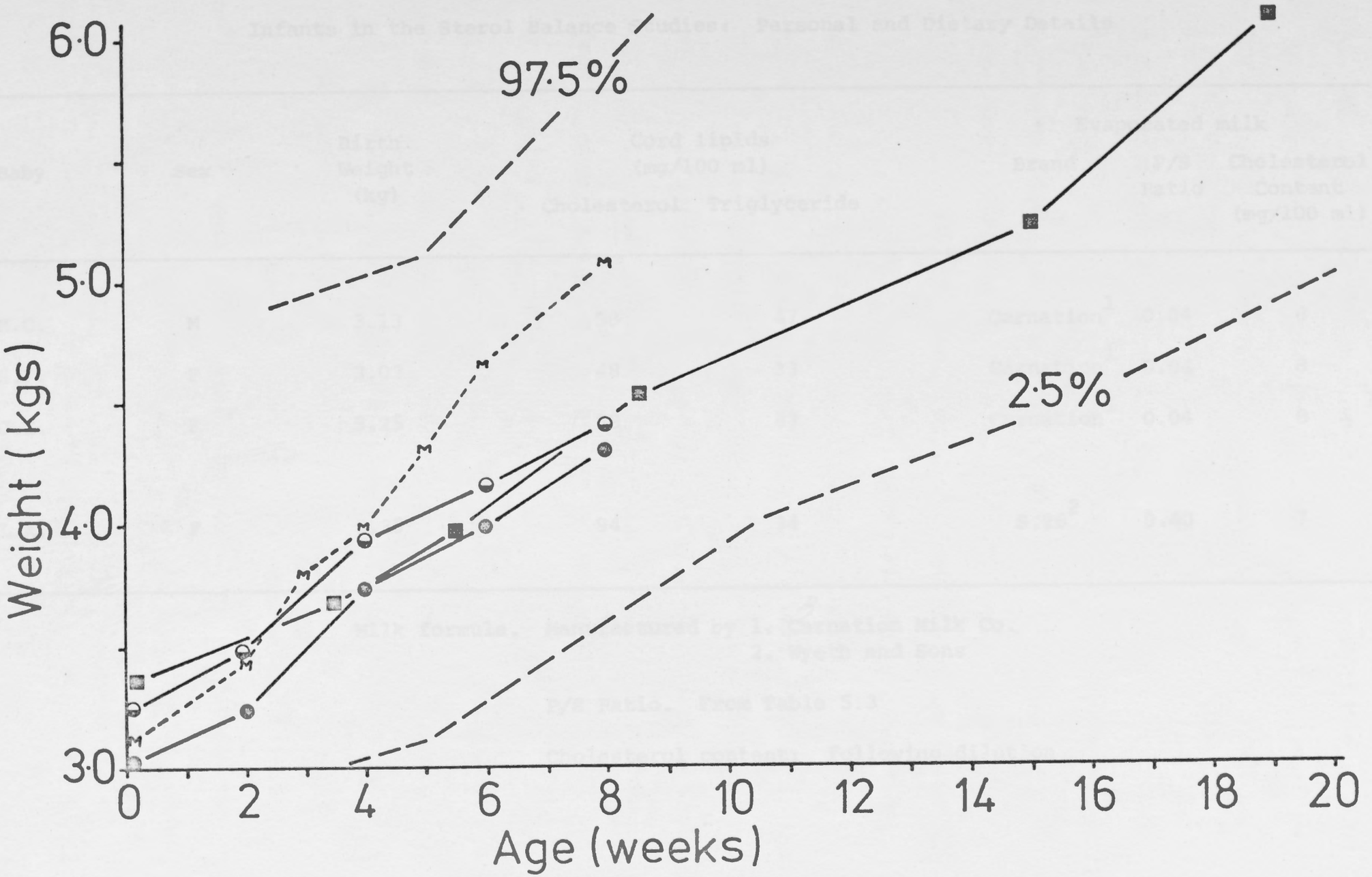

97·5%
2·5%
6·0
5·0
4·0
3·0
Weight (kgs)
0
2
4
6
8
10
12
14
16
18
20
Age (weeks)

TABLE 5.2

Infants in the Sterol Balance Studies: Personal and Dietary Details

Baby	Sex	Birth Weight (kg)	Cord lipids (mg/100 ml)		Evaporated milk		
			Cholesterol	Triglyceride	Brand	P/S Ratio	Cholesterol Content (mg/100 ml)
M.C.	M	3.13	58	47	Carnation[1]	0.04	8
S.F.	F	3.03	48	33	Carnation[1]	0.04	8
J.S.	F	3.25	58	47	Carnation[1]	0.04	8
L.K.	F	3.35	94	34	S.26[2]	0.40	7

Milk formula. Manufactured by 1. Carnation Milk Co.
2. Wyeth and Sons

P/S Ratio. From Table 5.3

Cholesterol content: following dilution

powder* was added to the soy milk. The quantity was calculated to approximate that in the evaporated milk being drunk by the 3 other infants at the same age. The purpose of this study was to determine the effect of dietary cholesterol in the presence of a high P/S ratio. Faeces were collected on days 5-10, 19-25, 41-47, 64-69, 115-120 and 127-133 inclusive.

B. DIETARY INTAKE

Both in hospital and at home, the mother kept a record of all milk drunk by the infant during each collection period. The amount of cholesterol ingested by the infant during the collection period was calculated from the total volume of milk drunk and the cholesterol concentration of the milk. The latter was ascertained by random sampling and measurement during the collection periods (Table 5.2). The only supplementation of diet of the first 3 infants was with small quantities of cereal and "fruit gel".** In the latter part of the studies, baby L.K. received daily supplements of 2.00 g egg yolk powder, containing 57.4 mg of cholesterol which was suspended in one-half of the soy milk ration. The bottle was then washed out with the other half.

C. SAMPLE COLLECTION

1. Blood Cord blood was collected at delivery for cholesterol measurements. Subsequent samples were collected from a heel prick on day 5 and at the beginning and end of each collection period. The infant was not necessarily fasting.

2. Faeces Faeces were collected from the infants using inert nappy liners (Johnson & Johnson, Pty. Ltd.) throughout the collection periods. Soiled liners were placed immediately into plastic bags and the faeces

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*Egg yolk powder: spray dried egg yolk, manufactured by The Egg Marketing Board for the State of New South Wales. Code YS-52, Batch No. 152/208. Grade A. Cholesterol content 2735 my per 100 g powder.

**Commercial product consisting of fruit jelly, marketed by baby food manufacturers.

deep frozen.

D. LABORATORY METHODS

1. Measurement of Plasma and Milk Cholesterol The plasma cholesterol was extracted directly into redistilled isopropanol. The method for measurement for small plasma samples has been described in Chapter 4.

Milk cholesterol was extracted by saponification of a 2 ml aliquot with 2 mls of 10 N sodium hydroxide and 18 mls of 95% ethanol, refluxing for 1 hour over a steam bath. On cooling, 10 mls of water was added, and the cholesterol extracted with petroleum ether (B.P. 60-80°C). An aliquot of this was dried down and redissolved in redistilled isopropanol.

Cholesterol was measured colorimetrically in a Technicon Auto Analyser II (Chapter 2).

2. Neutral Sterol and Bile Acid Extraction and Analysis A marker was not used in these studies. To minimize any irregularity in faecal flow collections were carried out continuously for 6-7 days. The totality of faecal collection was checked by measuring the plant sterol content of the faeces during the soy milk periods: recovery was found to be 101% and 115% in 2 children. This finding also excludes significant degradation of neutral sterols as described with liquid formulae diets in adults (Grundy *et al*, 1968).

The nappy liners were removed from the frozen faeces. Seven day pools were homogenized with water, and duplicate aliquots of about 2 mls were taken for neutral sterol and bile acid extraction and analysis. The method used was that of Miettinen *et al* (1965) and Grundy *et al* (1965), as described in Chapter 3.

3. Milk Fatty Acid Profile and P/S Ratio Samples of milk were analyzed for their total fatty acid composition according to the method described in Chapter 6, following extraction of 0.2 ml into chloroform: methanol (v:v); 2:1. The polyunsaturated to saturated (P/S) ratio was calculated from the sum of C18:2 plus C18:3 divided by the sum of

saturated C12 to C16 inclusive. Stearic acid (C18:0) was excluded because it has little effect on plasma cholesterol concentration in man (Keys *et al*, 1965c). The fatty acid profiles of commonly used infant milk formulae are shown in Table 5.3.

TABLE 5.3

Fatty Acid Composition of Milk Formulae

Commonly Used During Infancy

Milk Formula	Fatty Acid Composition (% Total fatty acids)									P/S Ratio
	C10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Cow's milk	1	2	9	27	4	10	32	4		0.08
Carnation[1]		0.2	9.9	36.2	2.4	17.4	32.0	1.9		0.04
S.26[2]	1	19.5	9.4	14.9	1.1	8.3	27.7	17.7		0.40
S.M.A.[2]	1.4	19.3	9.8	17.3	1.8	9.5	30.8	19.4		0.40
Lactogen[3]		0.7	10.1	34.4	2.6	16.4	33.3	2.4		0.04
Pro Sobee[4]				14.4		5.9	25.1	51.8	8.7	4.2

Milk formulae: Evaporated cow's milk manufactured by;

1. Carnation Milk Company
3. The Nestle Company (Aust.) Ltd.

Evaporated cow's milk with vegetable oil additives;

2. Wyeth and Sons

Soy bean extract;

4. Mead Johnson

RESULTS

A. PLASMA CHOLESTEROL

The plasma cholesterol level of the infants doubled during the first 5 days, and thereafter responded to changes in the diet as shown in Table

5.4. The high P/S ratio, cholesterol-free milk (Pro Sobee) reduced the plasma cholesterol level of all infants and this was reversed when the original formula was reintroduced.

TABLE 5.4

Plasma Cholesterol Concentration With Dietary Change:

Individual and Mean Results

Milk Formula	Age of Infants	Plasma Cholesterol (mg/100 ml) Infant	M.C.	S.F.	A.S.	L.K.	Mean (± S.E.M.)
	Birth		58	48	58	94	65 ± 10.1
Carnation S26	5 days		133	100	122	169	131 ± 14.4
Carnation S26	3 weeks		133	200	122	131	147 ± 18.0
Pro Sobee	5 weeks		106	100	118	111	109 ± 3.8
Carnation	8 weeks		153	140	125		139 ± 8.1
Pro Sobee + egg yolk	16 weeks					120	

In the single infant, L.K., who was maintained on Pro Sobee, the plasma cholesterol level remained low on the high P/S, cholesterol-free formula. Addition of egg-yolk cholesterol increased the plasma cholesterol, though not to the level seen with the cholesterol-containing formula, S26,

the latter having a P/S ratio of 0.40 and a cholesterol content equivalent to the amount added as egg yolk.

B. NEUTRAL STEROL AND BILE ACID EXCRETION

All results have been expressed in terms of mg/kg/day in an attempt to make the data comparable from one infant to another, and to take into account changes with growth.

1. Sterol Excretion During Infancy During the first 6-10 days of life, the infants excreted 1.7 mg/kg/day of bile acid and had a net sterol output of 6.3 mg/kg/day (Table 5.5). The output of both doubled over the next 2 weeks (bile acids $P < 0.001$; net sterol excretion, $p < 0.01$), so that by 3 weeks of age bile acid excretion was comparable to that published for adults (cf Miettinen, 1973). Bile acid and net sterol excretion did not change significantly thereafter on comparable diets at 8 weeks of age.

Throughout the studies, most of the neutral sterol recovered in the faeces was in the form of cholesterol. Only one infant (A.S.) excreted significant amounts of coprostanol, which amounted to one-quarter of the total neutral sterols measured whilst on the soy bean milk. This is in contrast to the adult, in whom most excreted cholesterol has undergone microbiological transformation, most commonly to coprostanol.

2. The Effect of Changing the P/S Ratio and the Cholesterol Content of Milk Three infants took part in a cross-over study of evaporated milk (P/S, 0.04; cholesterol content, 7 mg/100 ml), and soy bean milk (P/S, 4.20; cholesterol free). A fourth infant was fed the soy bean milk for 2 months, in order to study the longer term effects of high P/S ratio on sterol excretion, and cholesterol was also later added to the formula to examine the interaction of high P/S ratio and cholesterol intake.

The combined effect of withdrawing cholesterol and increasing the P/S ratio resulted in a 2 to 5 fold increase in net sterol output in 3

TABLE 5.5

Faecal Sterol and Bile Acid Excretion in the Neonate and Young Infant:

The Effect of Age and Dietary Change

Infant	Milk Formula	Weight (kg)	Sterol Excretion (mg/kg/day) Neutral Sterols	Bile Acids	Total Sterols	Cholesterol Intake (mg/kg/day)	Net Sterol Output (mg/kg/day)
Neonatal Period (6-10 days of age)							
M.C.	Carnation	3.13	15.3	1.1	16.4	15.0	1.4
S.F.	Carnation	3.03	24.0	2.2	26.2	15.2	9.0
A.S.	Carnation	3.25	17.4	1.6	19.0	12.6	6.4
L.K.	S.26	3.35	13.4	1.8	15.2	6.7	8.5
Mean ± S.E.M.			17.5 ± 2.3	1.7 ± 0.2	19.2 ± 2.5		6.3 ± 1.7
Three Weeks of Age							
M.C.	Carnation	3.62	16.6	2.5	19.3	16.6	4.1
S.F.	Carnation	3.48	28.7	3.6	32.2	13.5	18.7
A.S.	Carnation	3.67	16.9	4.6	21.5	14.0	8.2
L.K.	S.26	3.67	26.2	3.4	29.7	9.7	20.2
Mean ± S.E.M.			22.1 ± 3.1	3.5 ± 0.4	25.7 ± 3.1		12.8 ± 3.9

Continued

TABLE 5.5

(Continued)

Infant	Milk Formula	Weight (kg)	Sterol Excretion (mg/kg/day) Neutral Sterols	Bile Acids	Total Sterols	Cholesterol Intake (mg/kg/day)	Net Sterol Output (mg/kg/day)
Five Weeks of Age							
M.C.	Pro Sobee	4.30	18.4	3.4	21.9		21.9
S.F.	Pro Sobee	3.85	26.2	23.2	49.4		49.4
A.S.	Pro Sobee	4.03	19.4	10.8	30.0		30.0
L.K.	Pro Sobee	3.91	12.8	4.3	17.1		17.1
Mean ± S.E.M.			19.2 ± 2.7	10.4 ± 4.6	29.6 ± 7.1		29.6 ± 7.1
Eight Weeks of Age							
M.C.	Carnation	5.02	21.5	1.0	22.5	17.0	5.4
S.F.	Carnation	3.98	23.1	1.7	24.9	14.4	7.5
A.S.	Carnation	4.14	20.5	5.6	26.1	14.0	12.1
Mean ± S.E.M.			21.7 ± 0.8	2.8 ± 1.4	24.5 ± 1.1		8.3 ± 2.0

TABLE 5.6

Faecal Sterol and Bile Acid Excretion in One Young Infant:

The Effect of Prolonged Feeding of a High P/S Diet and of Cholesterol

Milk Formula		Duration (wks)	Age (wks)	Weight (kg)	Plasma Cholesterol Concentration (mg/100 ml)	Sterol Excretion (mg/kg/day)			Dietary Cholesterol (mg/kg/day)	Net Sterol Excretion (mg/kg/day)
Name	P/S Ratio					Neutral Sterols	Bile Acids	Total Sterols		
S.26	0.40	3	3	3.7	131	26.2	3.4	29.7	9.7	20.2
Pro Sobee	4.2	2	5	3.9	111	12.2	4.3	17.1		17.1
Pro Sobee	4.2	7	10	4.8	98	13.6	4.6	18.2		18.2
Pro Sobee plus egg yolk	4.2	2	16	5.2	120	30.4	9.1	39.5	10.5	29.0
Pro Sobee plus egg yolk	4.2	4	18	5.6	113	35.6	5.0	40.6	9.8	30.8

of the infants (Table 5.5). This was statistically significantly higher for the group compared to the preceding diet ($p < 0.02$) (Figure 5.2). The infant who did not respond (L.K.) had already been receiving S26 formula milk, which has a P/S ratio of 0.40 compared with 0.04 for the evaporated cow's milk (Table 5.3). A further decrease in plasma cholesterol did occur however (Table 5.4). The bile acid excretion increased in all infants (Table 5.5, Figure 5.2). There was wide variation in response, from 26% (L.K.) to 550% (S.F.).

When the soy bean milk was replaced by the initial evaporated milk, all 3 infants demonstrated a decrease in net sterol excretion ($p < 0.01$) and bile acid excretion (Table 5.5, Figure 5.2).

Both bile acid and neutral sterol excretion remained elevated in baby L.K., in whom the soy bean formula was continued for 2 months (Table 5.6, Figure 5.3). The addition of cholesterol to the diet resulted in a doubling of the bile acid excretion and a 60% increase in net sterol output during the first 2 weeks. The plasma cholesterol level increased approximately 20%. Over the next 2 weeks, the bile acid excretion returned towards previous levels, but net sterol excretion remained elevated, indicating that the additional sterol was being re-excreted in the neutral sterol fraction.

DISCUSSION

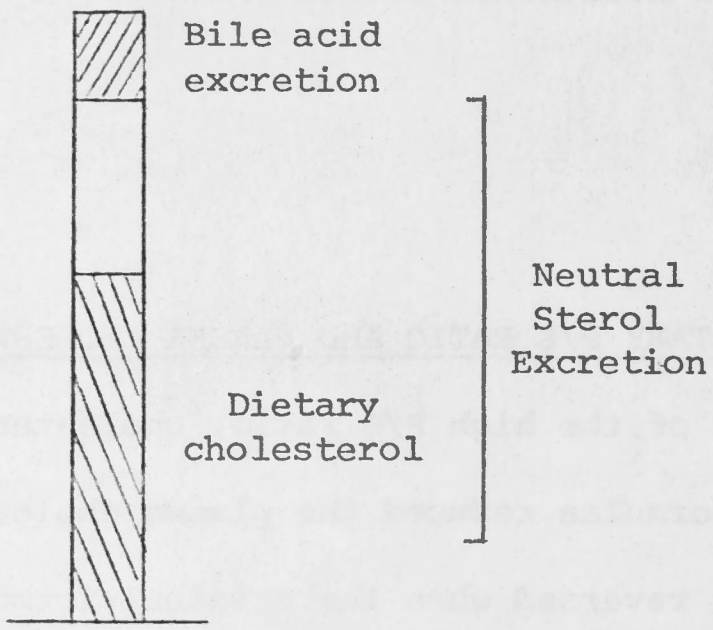
A. THE DIETARY P/S RATIO AND PLASMA CHOLESTEROL DURING INFANCY

Substitution of the high P/S ratio, cholesterol-free milk for the cow's milk based formulae reduced the plasma cholesterol level in all 4 infants. This was reversed when the original formula (lower P/S ratio and containing cholesterol) was reintroduced. Addition of an equivalent amount of cholesterol to the soy bean formula increased the plasma cholesterol level in the one infant, but not to the level seen previously on the lower P/S ratio formula.

FIGURE 5.2 Faecal Sterol and Bile Acid Excretion in the Neonate and Young Infant: The Effect of Age and Dietary Change

The faecal excretion of neutrol sterols and bile acids was measured at 6-10 and 19-25 days of age in 4 infants and in 3 infants at 50-56 days of age, whilst on a cholesterol-containing, low P/S ratio milk. All 4 infants were switched to a cholesterol-free, high P/S ratio formula at 26 days of age.

The plasma cholesterol concentration of the group (mean ± S.E.M.) during each study is shown above the histograms designed to represent



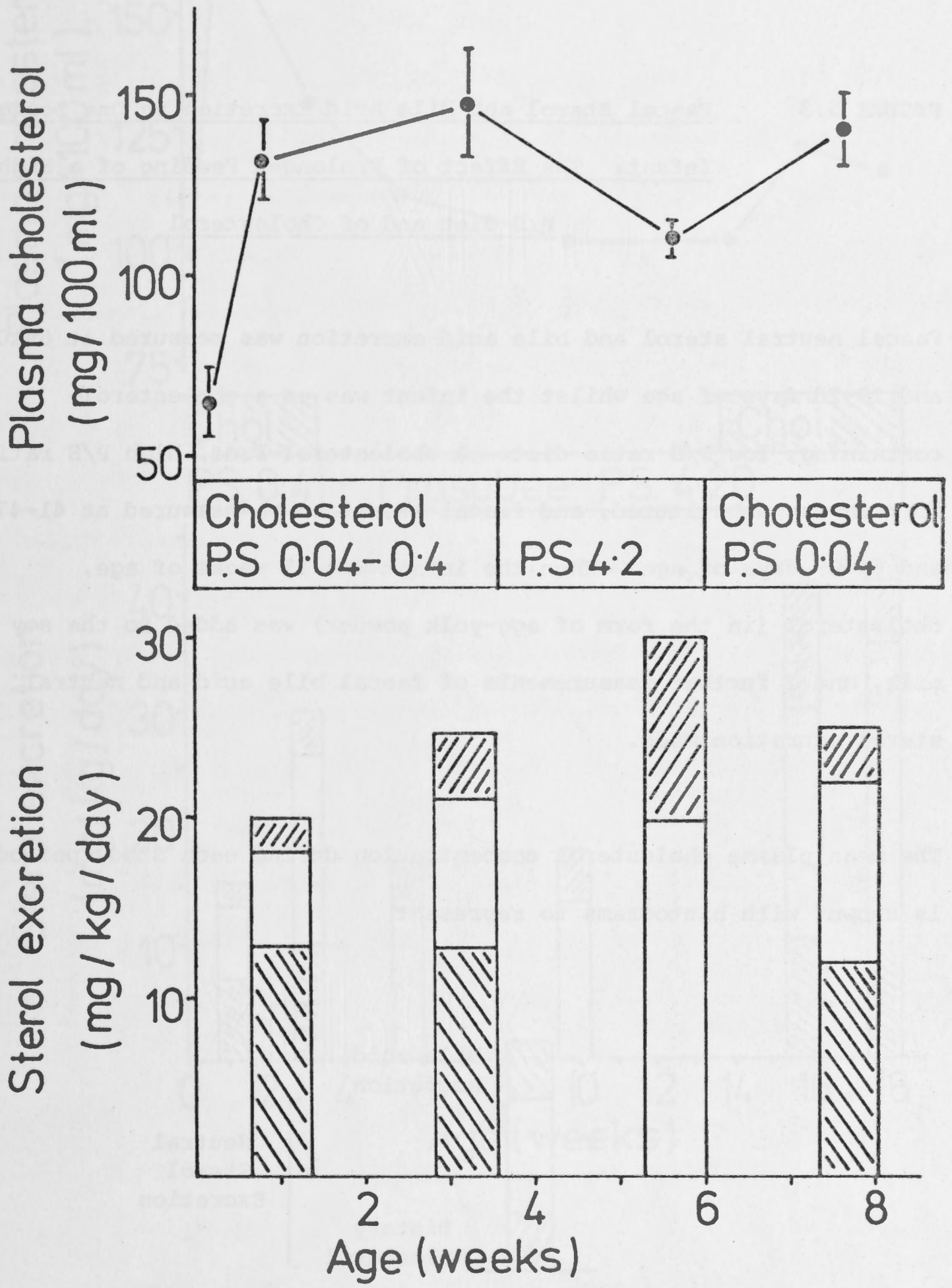
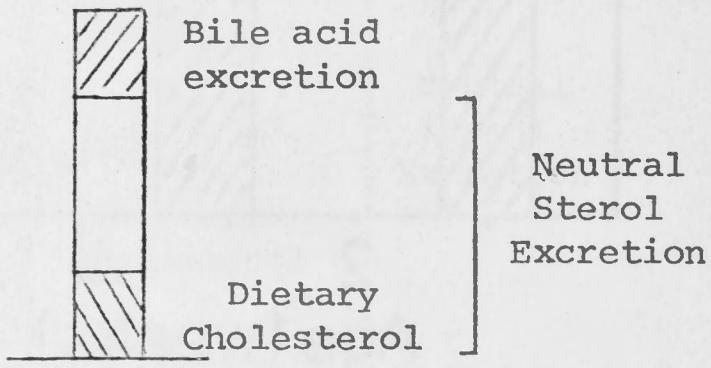
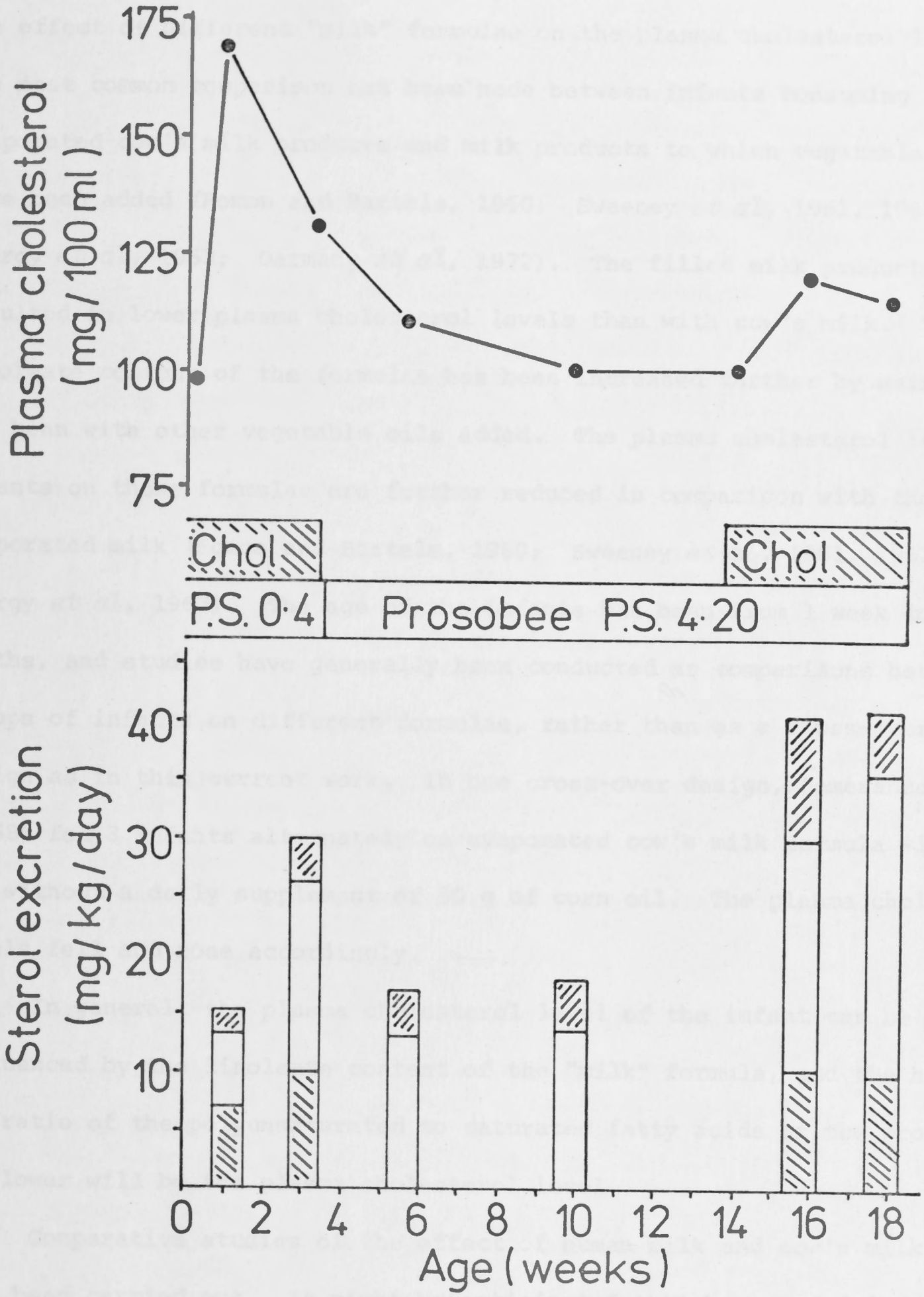

Plasma cholesterol
(mg/100ml)
150
100
50
Cholesterol
P.S 0·04-0·4
P.S 4·2
Cholesterol
P.S 0·04
Sterol excretion
(mg/kg/day)
30
20
10
2
4
6
8
Age (weeks)

FIGURE 5.3 Faecal Sterol and Bile Acid Excretion in One Young Infant: The Effect of Prolonged Feeding of a High P/S diet and of Cholesterol

Faecal neutral sterol and bile acid excretion was measured at 6-10, and 19-25 days of age whilst the infant was on a cholesterol-containing, low P/S ratio diet. A cholesterol-free, high P/S ratio formula was substituted, and faecal excretion re-measured at 41-47 and 64-69 days of age. When the infant was 15 weeks of age, cholesterol (in the form of egg-yolk powder) was added to the soy milk, and 2 further measurements of faecal bile acid and neutral sterol excretion made.

The mean plasma cholesterol concentration during each study period is shown, with histograms to represent




Plasma cholesterol (mg/100 ml)
175
150
125
100
75
Chol
Chol
P.S. 0·4
Prosobee P.S. 4·20
Sterol excretion (mg/kg/day)
40
30
20
10
0
2
4
6
8
10
12
14
16
18
Age (weeks)

Numerous short-term studies in the young infant have demonstrated the effect of different "milk" formulae on the plasma cholesterol level. The most common comparison has been made between infants consuming evaporated cow's milk products and milk products to which vegetable oils have been added (Fomon and Bartels, 1960; Sweeney *et al*, 1961, 1962; György *et al*, 1963; Darmady *et al*, 1972). The filled milk products resulted in lower plasma cholesterol levels than with cow's milk. The linoleate content of the formulae has been increased further by using soy bean with other vegetable oils added. The plasma cholesterol level of infants on these formulae are further reduced in comparison with those fed evaporated milk (Fomon and Bartels, 1960; Sweeney *et al*, 1961, 1962; György *et al*, 1963). The age of the infants has been from 1 week to 6 months, and studies have generally been conducted as comparisons between groups of infants on different formulae, rather than as a cross-over design as in this current work. In one cross-over design, Pomeranze *et al* (1958) fed 3 infants alternately on evaporated cow's milk formula with and without a daily supplement of 50 g of corn oil. The plasma cholesterol levels fell and rose accordingly.

In general, the plasma cholesterol level of the infant can be influenced by the linoleate content of the "milk" formula, and the higher the ratio of the polyunsaturated to saturated fatty acids of the product, the lower will be the plasma cholesterol level.

Comparative studies of the effect of human milk and cow's milk have also been carried out. It might be anticipated that breast-fed infants would have lower plasma cholesterol levels than those fed cow's milk, since the linoleate content of human milk (Insull and Ahrens, 1959) is approximately twice that of evaporated milk (Table 5.3). However, among infants 6 weeks of age and older, breast-fed infants either had higher (Fomon and Bartels, 1960; McKerrow, 1961; Darmady *et al*, 1972) or similar (Woodruff *et al*, 1964) plasma concentrations to those being fed cow's milk.

At one week of age no difference was apparent (Darmady *et al*, 1972). One exception has been published (György *et al*, 1963); premature infants up to 13 weeks of age being fed pooled human milk had lower plasma cholesterol levels than those fed evaporated milk. However, the former infants were hypoproteinaemic and showed poor weight gain and the difference in plasma cholesterol might have been due to deficient calories. In a second group of premature infants supplementation of the breast milk with casein resulted in plasma cholesterol and protein levels comparable to those on cow's milk.

During the perinatal period, lipid absorption is much less efficient than in the adult (Fomon *et al*, 1970). The percentage of fat absorbed from cow's milk is smaller than from human milk in both full term neonates (Fomon *et al*, 1970) and premature infants (Signer *et al*, 1974). Absorption of lipid particularly palmitic and stearic acids by the neonate is highest when palmitate is predominantly present in the β-position of the triglyceride molecule (Filer *et al*, 1969). This is the case with human milk fat, while in butter fat palmitate is equally distributed among the 3 positions (Freeman *et al*, 1965). Long chain polyunsaturated fatty acids and short chain saturated fatty acids seem equally well absorbed under most circumstances (Fomon *et al*, 1970). The linoleate absorbed is proportional to the content of the milk formula (Barness *et al*, 1974). If the plasma cholesterol concentration in an infant is dependent upon the relative caloric contributions of saturated and unsaturated fatty acids, as in the adult (Keys *et al*, 1965a), then the better absorption of palmitate from human milk may discount any advantage gained from the increased linoleic acid content of human relative to cow's milk.

B. STEROL METABOLISM

1. Excretion of Bile Acid by the Neonate The faecal excretion of bile acid in the neonates at 6-10 days of age was 1.7 mg/kg/day,

increasing to 3.5 mg/kg/day during the first 3 weeks of life. Few values of bile acid excretion in childhood have been reported. Measurements using an enzymatic method have yielded values of 7.3 mg/kg/day for premature infants (n=16) and 6.1 mg/kg/day for children aged 4 months to 5 years (n=18) (Weber *et al*, 1972). Using a similar enzymatic method, but fluorometric rather than colorimetric assay, Signer *et al* (1974) obtained values of 21.0 mg/kg/day and 36.2 mg/kg/day in 2 groups of premature infants being fed human milk and cow's milk respectively. These latter values are considerably higher than those obtained in the current work, which compare more favourably with other studies in which the daily bile acid excretion was 10-85 mg/day in 16 children, aged 2 months to 14 years of age (Leyland, 1970). This latter study utilized gas chromatographic techniques, and excretion was found to correlate with body weight. Bile acid excretion is reduced in young girls with anorexia nervosa, and may reflect diminished food intake and decreased body weight (Nestel, 1974). Values obtained in adults have been 3.8 mg/kg/day (Miettinen, 1973) and 3.7 mg/kg/day in normolipidaemic subjects (Nestel and Hunter, 1974).

2. Sterol Balance and Metabolism in the Neonate and Infant Net sterol excretion was low in the first 6-10 days of life and doubled over the next 2 weeks while the infants were being fed the same milk formula. It did not change further, provided the diet was not changed.

Ideal studies of cholesterol turnover demand maintenance of a steady state, particularly with regard to constant sterol ingestion and body weight. Obviously the latter requirement cannot apply in studies of growing infants. The average increase in body weight per week was about 0.15 kg or 5%. In a true steady state, cholesterol synthesis equals the excretion of bile acids plus neutral sterols when the diet is cholesterol free. When cholesterol is present in the diet, synthesis equals total sterol excretion minus dietary cholesterol. Since in the

present studies a steady state cannot be assumed in view of the continuing growth of the infant and its expanding cholesterol pool, the difference between total sterol excretion and cholesterol intake is simply the net sterol output. This represents newly synthesized cholesterol and re-excreted dietary cholesterol in those infants consuming cholesterol. A change in net sterol output may therefore reflect changes in synthesis, in re-excretion or in both. Although one cannot distinguish between these, certain assumptions can and will be made on the basis of findings in adults studied under steady state conditions.

The rate of synthesis of cholic acid in the mature newborn is 22.7 ± 4.1 mg/day (mean ± S.E.M.) or 110 ± 20 mg/m^2/day (Watkins *et al*, 1973a). Expression of data in the literature has often been in terms of surface area, since comparison of metabolic data between small and large animals and infants and adults correlates better with body surface area than body weight (Kleiber, 1947). The data of Watkins *et al* (1973a) was obtained by measurement of the dilution in bile of orally administered deuterium labelled cholic acid. This also requires steady state conditions and therefore the validity of the data is in doubt. In premature infants of 34-36 weeks' gestation, cholate synthesis was reported as 6.3 ± 1.2 mg/day, and that of chenodeoxycholate 1.6 ± 0.1 mg/day (Watkins *et al*, 1973b). The cholate pool sizes were estimated to be 41.7 ± 7.4 mg and 15 ± 1.6 mg in the mature and premature infants respectively, with that of chenodeoxycholate being 3.8 ± 0.7 mg in the premature infant. The rate of synthesis was therefore very high compared to the total pool size. From the current study then, although the values for the newborn are less than in the adult, this difference tends to disappear within 3 weeks of birth (Table 5.5).

Studies in the foetal dog have demonstrated that a well-developed enterohepatic circulation exists *in utero* (Lester *et al*, 1972), but the pattern of bile acid absorption is different to that in fully-grown

animals (Smallwood *et al*, 1970). Absorption of taurocholate from the jejunum is nearly as efficient as from the ileum. The suckling rat has an efficient conservation of bile acids, with very slow rates of faecal excretion (Harris *et al*, 1967). This is coupled with low rates of mevalonate synthesis, which in association with the *in vitro* inhibition of mevalonate synthesis by addition of taurocholate, has been interpreted as feed-back inhibition of cholesterol synthesis by bile acids (Harris *et al*, 1967). Weaning leads to an increase in bile acid excretion, together with an increase in cholesterol synthesis (Harris *et al*, 1967; McNamara *et al*, 1972), which has been attributed to changes in the propertions of either fat, protein or carbohydrate in the diet (Harris *et al*, 1966; Carroll *et al*, 1973). Similar changes in synthesis are produced by interruption of the enterohepatic circulation, either surgically (Harris *et al*, 1967; McNamara *et al*, 1972) or with bile acid binding resins (Harris *et al*, 1967) in the suckling or in the adult animal (Huff *et al*, 1963; Shefer *et al*, 1968), as well as in man (Grundy *et al*, 1971). The mechanism of sequestration of bile acids appears to be important in the increased bile acid loss in fibrocystic disease (Weber *et al*, 1973). Greater excretion of bile acid has been demonstrated in premature infants fed a cow's milk substitute in comparison with human milk (Signer *et al*, 1974). This may have been due to the sequestration of bile salts by calcium soaps and glycerides which were less well absorbed in the premature than in the normal infants (Fomon *et al*, 1970; Watkins *et al*, 1974).

It seems then, that in the mature neonate during the first week of life, the size of the total bile acid pool and the faecal loss of total bile acids are about half that found in the adult, though the rate of cholate synthesis alone is very similar to the adult values. The premature infant has a lesser capacity to synthesize bile acids.

For reasons already discussed, the excretion of neutral sterols cannot be equated with cholesterol synthesis even during the periods of

cholesterol-free diets. In fact, the present studies do not include measurements in the absence of dietary cholesterol until the second month of life. Net sterol excretion then averaged 30 mg/kg/day, which is more than twice as high as in adults studied under steady state conditions on very low cholesterol diets (Connor *et al*, 1969; Quintão *et al*, 1971; Nestel and Hunter, 1974).

3. The Effects of Changing the Dietary P/S Ratio and Cholesterol on Sterol Metabolism in the Young Infant When cholesterol was removed from the diet and the P/S ratio of the formula increased, bile acid excretion increased in all subjects (Table 5.5). The net sterol excretion increased markedly in 3 out of the 4 infants (Table 5.5). The fourth infant (L.K.) had been on an artificial milk having a P/S ratio of 0.40 and may have already been responding to the stimulus of polyunsaturated fat, although her plasma cholesterol level did fall further with Pro Sobee and removal of dietary cholesterol (Table 5.6).

Two dietary factors must be considered, namely the effect of increasing the P/S ratio and the effect of reducing the cholesterol content. The actions of these two factors in lowering plasma cholesterol levels have been considered to be both independent and interdependent. Keys *et al* (1965b), Hegsted *et al* (1965) and Mattson *et al* (1972) support the significance and independence of the two. However, to obtain an adequate reduction in plasma cholesterol, the proportion of polyunsaturated fatty acids within the diet must be increased as the dietary cholesterol content is increased and Brown and Page (1965) and Brown (1970) have suggested an interdependence between the two. This is supported by studies of Connor *et al* (1961, 1964) and Erickson *et al* (1964), who showed that with cholesterol-free diets, even a 10-fold increase in P/S ratio (from 0.2 to 2.6) did not further lower the plasma cholesterol concentration. On the other hand, when in later studies the P/S ratio was raised to 4.7 the plasma cholesterol level was reduced even though the diet contained

no cholesterol (Connor *et al*, 1969).

The effect of dietary fat upon cholesterol and bile acid synthesis, and the basis for the reduction of plasma cholesterol which accompanies the use of polyunsaturated fatty acids, are not clear. Normal men eating no cholesterol increase their bile acid and neutral sterol excretion in response to an increase in the P/A ratio from <0.05 to 4.7, at least over a period of three weeks (Connor *et al*, 1969). Moore *et al* (1968), using experimental dietary periods of 16 days were also able to demonstrate an increase in faecal steroid excretion when the P/S ratio was increased. In the studies by Nestel *et al* (1973) the substitution of polyunsaturated for conventional ruminant products in the diet enhanced bile acid and neutral sterol excretion in normal subjects during the first 3 to 4 weeks. When the studies were carried on for 6 weeks, when the plasma cholesterol concentration had reached its new steady level, sterol excretion returned to control values (Nestel *et al*, 1974). This may explain the failure of other studies, notably those of Grundy and Ahrens (1970) to demonstrate a consistent increase in sterol excretion with polyunsaturated dietary fat. No difference in bile acid excretion could be shown in a comparison of corn oil and butter fat when the faecal collection was made only 4 days after the change of diet (Ali *et al*, 1966). The type of subject chosen for comparison of sterol excretion with polyunsaturated and more saturated fat diets may also be important. Most of the subjects studied by Grundy and Ahrens (1970) were genetically hypercholesterolaemic. It would seem that the response in terms of change in sterol excretion is better seen in normolipidaemic (Moore *et al*, 1968; Connor *et al*, 1969; Nestel *et al*, 1973) and hypertriglyceridaemic (Grundy and Ahrens, 1970; Grundy, 1974) rather than in hypercholesterolaemic subjects (Avignan and Steinberg, 1965; Spritz *et al*, 1965; Grundy and Ahrens, 1970). It should be noted that Grundy and Ahrens (1970) did find bile acid excretion to be increased in 5 out of 11 subjects "at some portion of the

unsaturated fat period", but they concluded that "changes in bile acid excretion *may* occur in association with decreasing plasma cholesterol on unsaturated fat diets, but such changes *need not* occur".

The increase in the infants' net sterol output during the cholesterol-free, high P/S formula milk feeding probably reflects 3 important changes. Firstly, the withdrawal of dietary cholesterol removes the suppression of cholesterol synthesis. Hepatic cholesterogenesis in man is under the control of direct feedback inhibition of dietary cholesterol (Bhattathiry and Siperstein, 1963; Fujiwara *et al*, 1965; Pawliger and Shipp, 1968), although this may not apply to all tissues. For instance cholesterol feeding did not produce suppression of the rate of acetate incorporation into digitonin-precipitable sterols in the human small intestine (Gamel and Dietschy, 1970). The activity of HMG-CoA reductase in cultured fibroblasts from normal human skin is regulated by the content of LDL and VLDL in the culture medium (Brown *et al*, 1973). The suppression is proportional to the amount of LDL which is bound to the cells and degraded (Brown and Goldstein, 1974). In cells derived from subjects homozygous for familial hypercholesterolaemia, HMG-CoA reductase was not similarly suppressed (Goldstein and Brown, 1973) probably because LDL binding to the cells was deficient (Brown and Goldstein, 1974). Increasing dietary cholesterol usually decreases cholesterol synthesis in man, as measured by sterol balance techniques (Grundy *et al*, 1969; Quintão *et al*, 1971), but exceptions have been reported (Quintão *et al*, 1971). The increased absorption of cholesterol with increased ingestion is also accompanied by increased re-excretion of cholesterol, and any accumulation of cholesterol within the body need not be reflected in plasma cholesterol levels (Quintão *et al*, 1971).

The second major factor to be considered as a cause for the increase in net sterol output is the re-excretion of endogenous cholesterol. In the infants on the soya bean formula, the presence of increased

quantities of plant sterol might have decreased the reabsorption of endogenous cholesterol as described by Grundy *et al* (1969) and Shefer *et al* (1973). Shefer *et al* (1973) noted that feeding sitosterol to rats not only markedly increased the specific activity of hepatic HMG-CoA reductase, but also raised that of cholesterol 7α-hydroxylase by 40%. Thus both cholesterol and bile acid synthesis were stimulated. This is analogous to partial interruption of the enterolymphatic circulation of cholesterol.

The third factor, namely a direct stimulation of biliary cholesterol and bile acid excretion by polyunsaturated fatty acids has already been discussed.

There is also a fourth point which should be considered in the infants. Milk from the rat (Harris *et al*, 1967; McNamara *et al*, 1972), cow and human (Boguslawski and Wróbel, 1974) inhibits cholesterogenesis. Activity is demonstrable both *in vivo* and *in vitro* in the rat, and the factor is heat stable. If such a factor were able to survive the processing of evaporated and other artificial milks, and if it were operative also in the human infant, then a change in the infant's formula from evaporated milk to the soya bean product might be accompanied by the removal of an inhibitor other than cholesterol.

One infant (L.K.) maintained a high sterol output throughout the 2 months on the soya bean, cholesterol-free formula. However, in the presence of 2 other factors known to increase cholesterol synthesis, it is not possible to claim that this demonstrated the maintenance of a polyunsaturated fat induced increase in sterol excretion. The addition of cholesterol to the Pro Sobee produced a short-term increase in bile acid excretion and a sustained rise in net sterol output. In human adults dietary cholesterol stimulates the re-excretion of endogenous cholesterol (Grundy and Ahrens, 1969; Quintão *et al*, 1971), but not of bile acids, which is the major excretory pathway in rat (Taylor and Gould,

1950; Wilson, 1962, 1964) and the dog (Abell *et al*, 1956; Pertsemlidis *et al*, 1973). Increased activity of cholesterol 7α-hydroxylase follows cholesterol feeding in rats (Boyd *et al*, 1968; Cohen *et al*, 1973). The increase in bile acid excretion was transient in baby L.K. and the absence of a sustained increase with continuing dietary cholesterol is consistent with findings in adults (Wilson and Lindsey, 1965). Since cholesterol synthesis would have been reduced due to the addition of the egg yolk cholesterol, the sustained increase in cholesterol excretion would represent enhancement of cholesterol re-excretion. In this infant, in contrast to the other 3, dietary cholesterol was fed together with polyunsaturated fat, both of which are known to stimulate cholesterol excretion. These 2 factors clearly outweighed any reduction in sterol excretion due to lessened synthesis.

CHAPTER 6

LACTATION STUDY

INTRODUCTION

Hypercholesterolaemia rarely presents as a clinical problem in childhood. However, the occurrence of hyperlipoproteinaemia in the paediatric age group of clinical practice is receiving wide attention, for here may lie the opportunity for prophylactic intervention in atherosclerosis. A proportion of children inheriting familial Type II hyperlipoproteinaemia may be detected at birth by measurement of umbilical cord plasma cholesterol (Glueck *et al*, 1971; Tsang *et al*, 1974b) or LDL-cholesterol (Kwiterovich *et al*, 1970, 1973; Greten *et al*, 1973).

Dietary cholesterol and the ratio between saturated and polyunsaturated fatty acids are important determinants of plasma cholesterol during infancy. Modification of these two factors may reverse hypercholesterolaemia to normal values (Glueck *et al*, 1972; Tsang *et al*, 1974b). It has been clearly demonstrated that the short term use of evaporated milk enriched with vegetable oils, or of soya bean milk products, lowers plasma cholesterol levels in infants (Sweeney *et al*, 1961; György *et al*, 1963; Lowe *et al*, 1964). Other studies have shown that feeding infants on human breast milk may result in plasma cholesterol levels as high, if not higher, than with cow's milk, despite the higher linoleic acid content of human milk (Fomon and Bartels, 1960; Woodruff *et al*, 1964; Darmady *et al*, 1972).

Two groups of children should be considered; those who have normal and those who have high plasma cholesterol levels. With a high rate of usage of commercial infant feeding formulas, estimated as being fed to approximately 80% of all infants in the U.S.A. (Fomon, 1971; Schubert, 1973), many infants are already being reared on a diet low in cholesterol and high in P/S ratio. It has been recommended that dietary modification be commenced in children at high risk during the first few years of life (Filer *et al*, 1972; Glueck *et al*, 1972; Mitchell *et al*, 1972). For the newborn hypercholesterolaemic infant, the use of an

artificial formula might seem preferable to human milk. However, the benefits of breast-feeding are numerous, and since the linoleic acid content of human milk can be increased (Insull *et al*, 1959), the use of human milk need not be ruled out.

This chapter describes the effect on the composition of human milk resulting from changes in the maternal diet. The ultimate aim of altering the lipid composition of the milk was to influence the feeding infant's cholesterol and fatty acid metabolism in order to decrease the plasma cholesterol.

METHODS

The effect of dietary composition on the lipid constituents of human milk was investigated in three separate studies. Each study included a period that resembled an average Australian diet containing 400-600 mg of cholesterol per day and fat that was rich in saturated fatty acids. This was compared with either a change in cholesterol content or fatty acid composition or both. All women were studied as out-patients in the free-living situation. The subjects were carefully instructed about weighing and preparation of food. They were asked to adhere rigidly to those items that provided the cholesterol and fat in their diet, as shown in Table 6.1. The remainder of their food intake was not controlled. Caloric intake was monitored by regular weighing, except in Study I, which was carried out within 5 weeks of delivery, when excess weight gained during pregnancy was being lost.

A. SUBJECTS

The women were between 22 and 31 years of age (mean 26.4 years); body weights ranged from 46 to 76 kg (mean 59.1 kg). Women taking part in the studies were selected on the basis of

(1) a normal pregnancy,

(2) an uncomplicated delivery,

TABLE 6.1

Estimated Cholesterol Content of Foods Used in the Lactation Studies

		Cholesterol content* (mg/100 g or mg/100 ml)
MEAT	Lean beef	94
	Lean lamb	98
	Cooked chicken	85
	Fish	80
DAIRY PRODUCTS	Full cream milk	14
	Skim milk	5
	Cream	100
	Yoghurt (low fat)	5
	Butter	250
	Cheddar cheese	100
	Cottage cheese	15
EGG	One yolk	230

*FOOTNOTE. The cholesterol content has been calculated from figures in Thomas and Carden (1970) and Watt and Merrill (1963).

(3) successful initiation and maintenance of lactation and

(4) a healthy baby.

No account was taken of parity (1-4) or previous breast-feeding experience.

B. EXPERIMENTAL DESIGN

Study I. A pilot study of 4 subjects consisted of 3 dietary periods of one week each as shown in Table 6.2, and was commenced only 10 days post partum. The estimated amounts of cholesterol and the foods from which this was derived are shown in Table 6.2. Diets A and B contained more cholesterol than did Diet C (approximately 575 mg and 110 mg respectively); Diet A was rich in saturated fatty acids, whilst Diets B and C had an increased content of polyunsaturated fatty acids.* All subjects received Diets A, B and C in that order.

Study II. This group consisted of 7 women who undertook the study for a total of 7 weeks. The effect of 2 diets was compared, one having an estimated cholesterol content of 575 mg and a high saturated fatty acid composition (Diet D), and the other (Diet E) having a much lower cholesterol content and a higher polyunsaturated fatty acid composition. The study was commenced 6 weeks post partum, by which time physiological involution had occurred. The diets were given in random order, each for 3 weeks, and the initial diet was retested for one week during the seventh week of the study.

Study III. A longer study of 3 women lasting 10-12 weeks compared the effects of two diets that were rich in either saturated or polyunsaturated fatty acids with similar intakes of cholesterol. The polyunsaturated diet was tested for 6 weeks and was preceded and followed by 1-3 weeks of the saturated diet. This study was also commenced when the infant was 6 weeks of age.

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*Polyunsaturated margarine and oils were used in place of butter in the more unsaturated Diets.

TABLE 6.2

Estimated Cholesterol Content of Diets Used in the Lactation Studies

STUDY I.

Diet	A. High cholesterol: Saturated fat		B. High cholesterol: Polyunsaturated fat		C. Low cholesterol: Polyunsaturated fat	
	300-360 g	meat	75-100 g	meat	75-100 g	meat
	45 g	cheese or cream	100 g	cottage cheese	100 g	cottage cheese
	600 ml	milk	210 ml	skim milk	210 ml	skim milk
	60 g	butter	100 g	low fat yoghurt	100 g	low fat yoghurt
			2	eggs		
Daily cholesterol		570 mg		570 mg		110 mg

STUDY II.

Diet	D. High cholesterol: Saturated fat		E. Low cholesterol: Varying saturation	
	100-120 g	meat	75-100 g	meat
	90 g	cheese	100 g	cottage cheese
	600 ml	milk	210 ml	skim milk
	30 g	butter	100 g	low fat yoghurt
	1	egg		
Daily cholesterol		580 mg		110 mg

Continued

TABLE 6.2

(Continued)

STUDY III.

Diet	F. Moderate cholesterol: Saturated fat		G. Moderate cholesterol: Polyunsaturated fat	
	100-120 g	meat	100-120 g	meat
	45 g	cheese	100 g	low fat yoghurt
	600 ml	milk	210 ml	skim milk
	60 g	butter	1	egg
Daily cholesterol	380 mg		345 mg	

C. COLLECTION OF SAMPLES

1. Mothers The composition of milk, especially that of its fat, changes both during a feed and in the course of a day (Hytten, 1954a,b). Obtaining representative samples of milk therefore poses difficulties, particularly in out-patients. In these studies, samples were collected at the same feed each day, either the first or second of the morning, and at the same point in relation to the feed, either before or after. The sample, 5-10 mls, was manually expressed from both breasts directly into a sterile container. One sample was collected on each of the last 3 days of each week. The 3 samples were analyzed separately. The milk was immediately refrigerated at 4°C and the lipids extracted within 72 hours. When longer storage periods were likely, the samples were immediately frozen. There was no difference between the results obtained from samples stored in these two ways. Lipid extracts of all milk samples were stored until completion of the study, then analyzed together.

Fasting blood samples were collected at the end of each week, and the plasma lipids extracted within 3 hours. Venepuncture of the antecubital vein was used, with the subject in a sitting position following a 5-10 minute rest period.

2. Infants Changes in the plasma cholesterol concentration were also determined in the infants of 8 of the women taking part in the studies, (5 out of the 7 in study II and all 3 in study III). The details of the frequency of blood sampling are shown in Table 6.3. The infants were weighed regularly and maintained normal growth rates (Figures 6.1 and 6.2). This was the simplest and most readily available way of assessing the infants' satisfactory progress and calorie intake, taking into account the variability in growth of individual infants week by week and the effect that any minor intercurrent illness may have. Although as shown in Table 6.3, the infants were predominantly breast fed throughout the studies, some supplementation was required. The

TABLE 6.3

Infants in the Lactation Studies — Personal, Dietary and Sampling Details

	Sex	Birth Weight (Kg)	Cord Cholesterol Concentration (mg/100 ml)	Plasma Sampling	Diet
STUDY I.					
M.B.	M	3.04	50	Weekly	Exclusively breast fed.
B.M.	F	3.36	—	1 sample each dietary period	Exclusively breast fed.
K.M.	M	3.47	68	Weekly	Cereals from 4 weeks of age, prior to commencement of study.
J.T.	F	3.96	58	Weekly	Predominantly breast fed.
G.W.	M	3.75	—	Weekly	Predominantly breast fed.
STUDY II.					
J.A.	M	3.55	58	Weekly	Predominantly breast fed.
L.R.	F	3.43	156	Weekly	Predominantly breast fed.
C.v.d.S	M	3.13	85	Weekly	Predominantly breast fed.

FIGURE 6.1 Growth of Male Infants During Lactation Studies

Infants were weighed prior to and during the lactation studies. Their growth is shown contained within the percentile limits recently obtained for an Australian population (NHMRC, 1974).

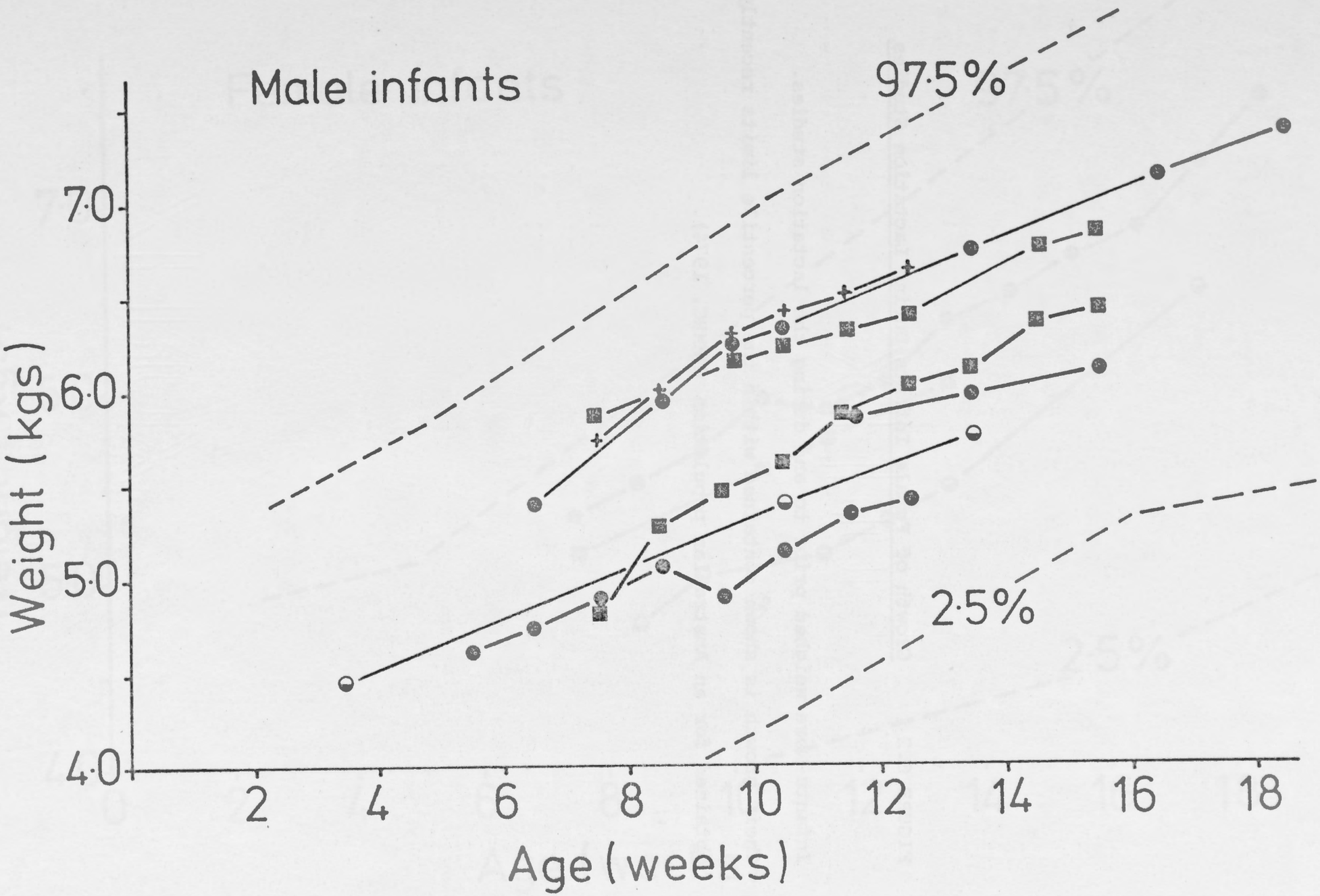
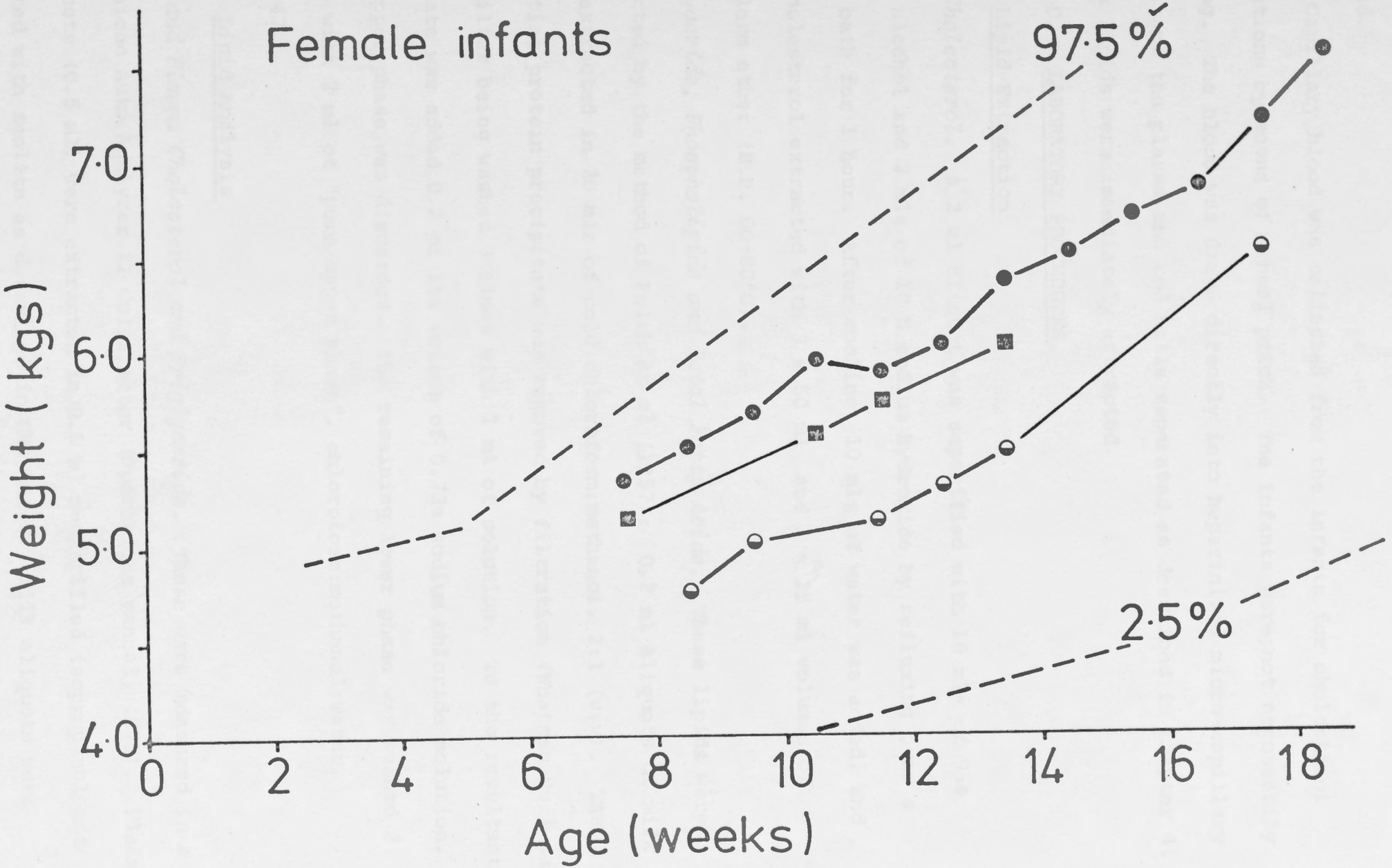

Male infants
97·5%
2·5%
Weight (kgs)
7·0
6·0
5·0
4·0
2
4
6
8
10
12
14
16
18
Age (weeks)

FIGURE 6.2 Growth of Female Infants During Lactation Studies

Infants were weighed prior to and during the lactation studies. Their growth is shown contained within the percentile limits recently obtained for an Australian population (NHMRC, 1974).


Female infants
97·5%
2·5%
Weight (kgs)
7·0
6·0
5·0
4·0
0
2
4
6
8
10
12
14
16
18
Age (weeks)

cholesterol intake from the supplemental foods was minimal, since only cereals mixed with expressed breast milk and fruit gel products were allowed.

Capillary blood was collected from the infants for cholesterol estimations by means of a heel prick. The infants were not necessarily fasting. The blood was drawn directly into heparinized micro-capillary tubes, and the plasma and red cells separated as described in Chapter 4. Plasma lipids were immediately extracted.

D. LABORATORY PROCEDURES

1. Lipid Extraction

Milk Cholesterol. A 2 ml aliquot was saponified with 18 mls of 95% ethyl alcohol and 2 mls of 10 N sodium hydroxide by refluxing over a steam bath for 1 hour. After cooling, 10 mls of water was added, and the cholesterol extracted with 3 x 50 ml and 1 x 25 ml volumes of petroleum ether (B.P. 60-80°C).

Triglyceride, Phospholipids and Total Fatty Acids. These lipids were extracted by the method of Folch *et al* (1957): 0.2 ml aliquots of milk were extracted in 20 mls of cold chloroform:methanol, 2:1 (v:v). The resulting protein precipitate was removed by filtration (Whatman No.1 paper), the filter being washed 3 times with 1 ml of solution. To the resultant filtrate was added 0.2 of its volume of 0.73% sodium chloride solution. The upper phase was discarded. The remaining lower phase was washed 3 times with 2 ml of "pure upper phase", chloroform:methanol:water, 3:48:47.

2. Lipid Analysis

Milk and Plasma Cholesterol and Triglyceride. These were measured in a Technican Auto Analyzer II Colorimeter (Operations manual, 1971). Plasma aliquots (0.5 ml) were extracted in 9.5 ml redistilled isopropanol and treated with zeolite as described in Chapter 2. Milk aliquots were taken from the lower phase of the chloroform:methanol extraction; the

latter was dried down under nitrogen, then made up to 10 mls with chloroform:methanol. A 1 ml aliquot was taken for triglyceride estimation, dried down under air, made up to 10 mls with isopropanol and treated with zeolite prior to assay. Milk cholesterol contained in 100 ml aliquots of the petroleum ether extract was evaporated to dryness, redissolved in 2 ml isopropanol, treated with zeolite and assayed.

Milk Phospholipids. The colorimetric estimation of inorganic phosphorus was used, according to the method of Bartlett (1959) and Morrison (1964). Milk inorganic phosphorus was removed during chloroform:methanol extraction of the lipids and subsequent washing of the lower phase. A 4 ml aliquot of the known extract volume (10 mls) was dried under air. Phospholipid in the sample was digested with concentrated sulphuric acid at 200°C for 30 minutes. A 30% solution of hydrogen peroxide was added for bleaching. Colour development was carried out with ammonium molybdate and Fiske-Subbarow reagent in a boiling water bath. Blanks consisting of concentrated sulphuric acid and standards of known phosphorus content (stock solution of di-potassium hydrogen or orthophosphate) were run simultaneously. They were read in a Beckman DB-G Grating Spectrophotometer, at 820 n.m.

Milk and Plasma Total Fatty Acids. The 5 ml aliquots of the chloroform: methanol extraction of milk or the total plasma extract were evaporated to dryness under nitrogen. Methylation of fatty acids was carried out with 5 ml 4% sulphuric acid in dry methanol for 12-18 hours at room temperature. The methyl esters were extracted by phase separation using 1 ml water and 4 ml heptane, and stored at 4°C in well sealed containers. Immediately prior to assay, the methyl esters were evaporated to 50-100 µl under nitrogen. Aliquots of 2-3 µl were injected into a Packard Series 7800 Gas Chromatograph, containing a 6 foot, coiled, glass column, packed with 13% ethylene glycol adipate, 80-100 mesh, on Gas-chrom P (Applied Science Lab., State Park, Pennsylvania, U.S.A.).

The following conditions operated:

Inlet temperature	190°C
Column temperature	185-190°C
Flame ionization detector temperature	200°C
Outlet temperature	195°C
Carrier gas (N_2) flow rate	40 cc/min
Hydrogen flow rate	40 cc/min
Air flow rate	400 cc/min

Standard mixtures of known methyl esters of fatty acids (N.I.H. mixtures C and F) were injected at the commencement of each days run, and at intervals during the day. Examples of the separation obtained for milk and plasma are shown in Figure 6.3.

The respective weights of the methyl esters were measured by estimation of the peak area on the chromatograph according to the formula;

Area α Peak Height x Retention time.

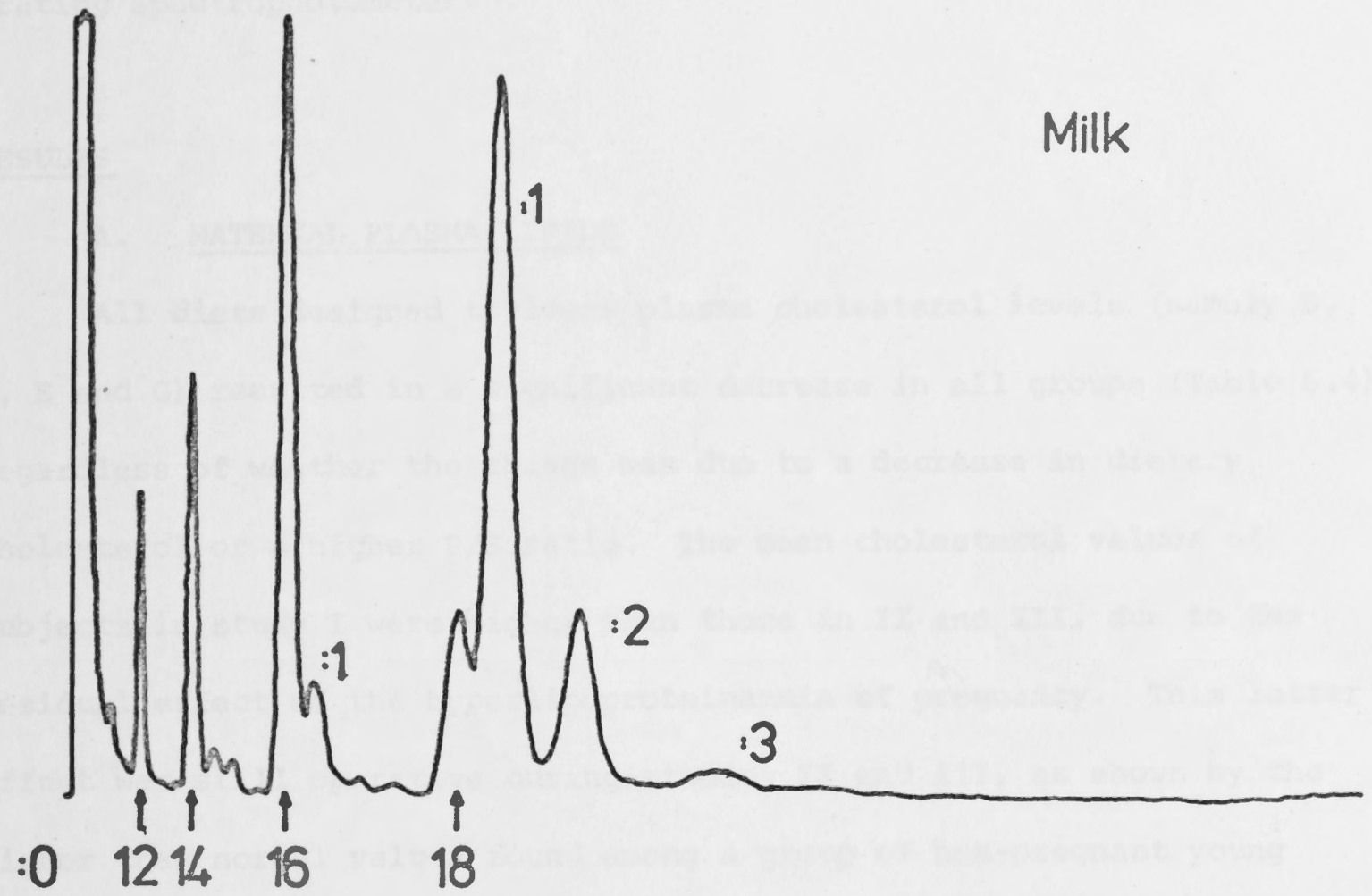
Each fatty acid has been expressed as a percentage of the total fatty acid content of the injected sample.

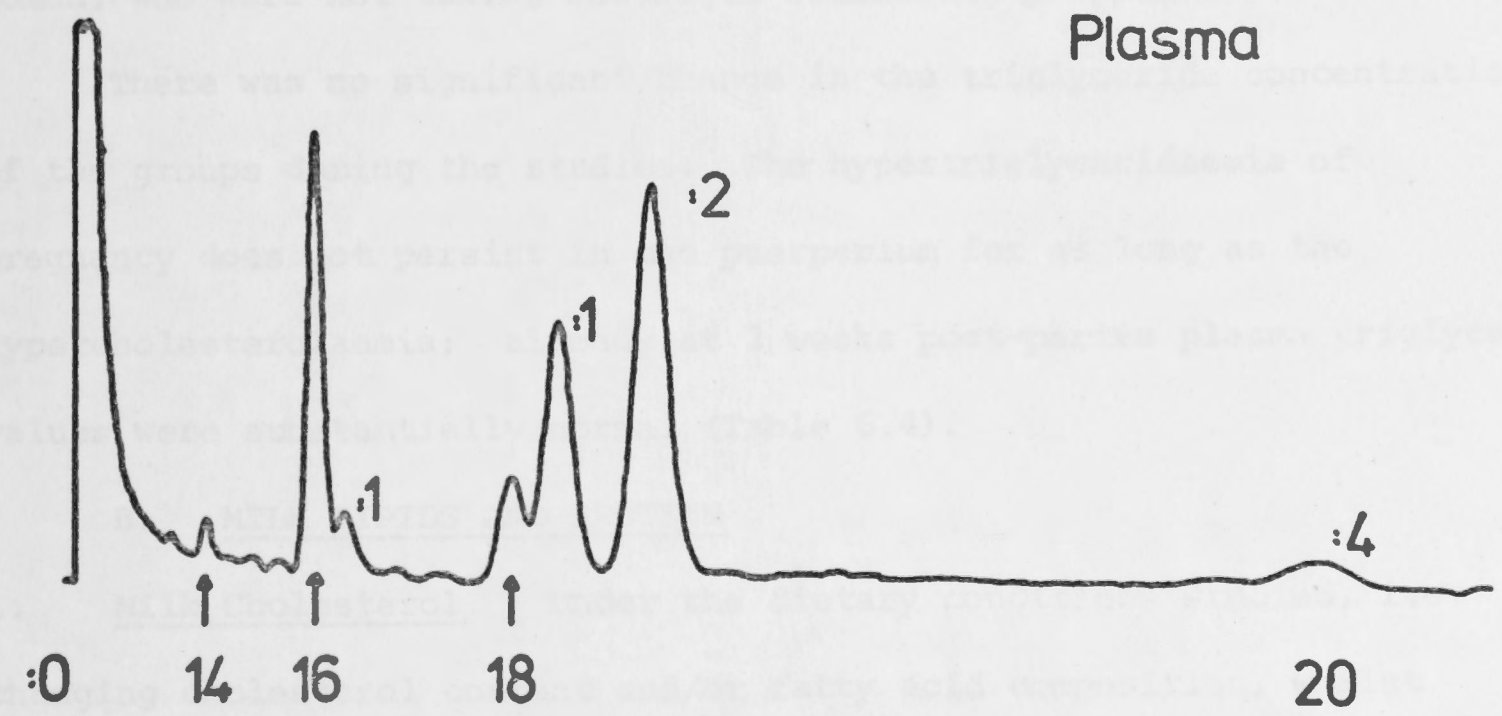
3. Milk Protein Estimation The protein content of milk samples was estimated using the colorimetric method developed by Lowry (1951). A 100 µl aliquot of milk was diluted 1 in 25 with distilled water, and 0.2 ml taken for protein estimation. Protein standards contained 25-50 µg of bovine serum albumin (Sigma Fraction V). Following colour development with Folin-Ciocalteau Phenol reagent, turbidity due to the presence of fat in the milk samples was cleared. This was done by

FIGURE 6.3 Separation of the Methyl Esters of the Total Fatty Acids of Human Plasma and Milk Using Gas Chromatography

Plasma and milk samples were extracted, methylated and analysed as described in the text.

The traces show typical patterns obtained. The length of the carbon chain and the number of unsaturated levels are indicated with the relevant peak.


Milk
:1
:1
:2
:3
:0
12
14
16
18


Plasma
:2
:1
:1
:4
:0
14
16
18
20

adding an equal volume of diethyl ether to the sample and mixing vigorously. Following phase separation, the lipid-containing upper ether layer was removed. Standards were subjected to the same treatment. The optical density of the samples was read at 740 nm on a Beckman DB-G Grating Spectrophotometer.

RESULTS

A. MATERNAL PLASMA LIPIDS

All diets designed to lower plasma cholesterol levels (namely B, C, E and G) resulted in a significant decrease in all groups (Table 6.4), regardless of whether the change was due to a decrease in dietary cholesterol or a higher P/S ratio. The mean cholesterol values of subjects in study I were higher than those in II and III, due to the residual effect of the hyperlipoproteinaemia of pregnancy. This latter effect was still operative during studies II and III, as shown by the higher than normal values found among a group of non-pregnant young women, who were not taking oestrogen-containing preparations.

There was no significant change in the triglyceride concentrations of the groups during the studies. The hypertriglyceridaemia of pregnancy does not persist in the puerperium for as long as the hypercholesterolaemia; already at 2 weeks post-partum plasma triglyceride values were substantially normal (Table 6.4).

B. MILK LIPIDS AND PROTEIN

1. Milk Cholesterol Under the dietary conditions studied, i.e. changing cholesterol content and/or fatty acid composition, whilst keeping the diet isocaloric, no significant change in milk lipid concentration could be demonstrated (Table 6.5). This is applicable to both individual subjects and to pooled group data, with only 2 exceptions. The change in lipid composition which accompanies the transition from colostrum to mature milk and which generally occurs during the first 2-3

TABLE 6.4

Maternal Plasma Cholesterol and Triglyceride Concentrations

During the Lactation Studies

Study	Diet			Plasma concentration (mg/100 ml)	
		Saturation of fat*	Cholesterol (mg/day)	Cholesterol	Triglyceride
I	A	S	570	349 ± 47**	116 ± 27
(n=4)	B	P	570	316 ± 38	102 ± 48
	C	P	110	276 ± 41	109 ± 37
II	D	S	580	297 ± 107	97 ± 25
(n=7)	E	P	110	260 ± 93	94 ± 34
III	F	S	380	226 ± 18	90 ± 33
(n=3)	G	P	345	206 ± 15	90 ± 34

Significant differences.	Cholesterol.	A-B	p = 0.062
		B-C	<0.001
		D-E	<0.001
		F-G	<0.001

*Saturation of fat: S - rich in saturated fatty acids
P - rich in polyunsaturated fatty acids

**Plasma concentration: mean ± S.D.

TABLE 6.5

Milk Lipid and Protein Concentrations

Study		Diet Saturation of fat*	Diet Cholesterol (mg/day)	Milk Concentration Cholesterol (mg/100 ml)	Triglyceride (g/100 ml)	Phospholipid (mg P_i/100 ml)	Protein (g/100 ml)
I	A	S	570	23.2 ± 5.9**	2.90 ± 0.99	5.72 ± 1.08	
(n=4)	B	P	570	19.4 ± 2.8	2.55 ± 0.86	4.74 ± 0.98	
	C	P	110	20.4 ± 2.5	3.35 ± 0.71	5.81 ± 2.18	
II	D	S	580	18.1 ± 2.7	3.42 ± 0.61	4.04 ± 0.71	0.89 ± 0.03
(n=7)	E	P	110	19.3 ± 3.6	3.57 ± 0.82	4.18 ± 0.91	0.91 ± 0.03
III	F	S	380	23.3 ± 2.3	4.11 ± 0.42		
(n=3)	G	P	345	21.3 ± 2.4	4.12 ± 0.56		

*Saturation of fat: S - rich in saturated fatty acids
P - rich in polyunsaturated fatty acids

**Milk concentration: mean ± S.E.M.

weeks of lactation, has been observed previously (Macy and Kelly, 1961). It consists of an increase in the total fat content of the milk and a decrease in the proportion of cholesterol, with only minor changes in the phospholipid moiety. The cholesterol content of milk from one subject fell from 31.8 mg/100 ml (Diet A) to 23.1 mg/100 ml (Diet B) and then further to 17.4 mg/100 ml when Diet C was introduced. This could be explained on the basis of milk maturation. However, her milk triglyceride values also decreased by 14% of the level with Diet A. This demonstrates a relationship between milk cholesterol and triglyceride which will be discussed in more detail below.

In no single individual, nor in any group, was a significant relationship found between the plasma cholesterol concentration and the cholesterol content of the milk. Despite significant changes in the plasma cholesterol levels brought about by dietary change, the cholesterol content of the milk could not be varied correspondingly. It is relevant to point out that the one hypercholesterolaemic woman did not produce milk with a higher cholesterol content than was found in the normocholesterolaemic women.

2. Milk Triglyceride Whilst there was wide variation in the triglyceride content of milk among the women, no significant changes were brought about by dietary change within the groups. The triglyceride content of milk varied from 1.3 g/100 ml to 6.8 g/100 ml and averaged 3-4 g/100 ml in the 3 studies (Table 6.5).

3. Milk Protein The protein content of milk remained constant throughout the studies, and showed little variation between the women.

4. Relationship Between Milk Cholesterol, Triglyceride and Phospholipid Strongly positive correlations were found between the various lipid constituents of milk (Table 6.6). The correlation coefficients were particularly high in studies II and III, i.e. during well established lactation. However, in transitional milk (study I) the relationships

TABLE 6.6

Inter-relationships Between the Concentrations of

Milk Cholesterol, Triglyceride and Phospholipid

Subject	Cholesterol - Triglyceride		Phospholipid - Triglyceride	
	Correlation coefficient (r)	Significance (p)	Correlation coefficient (r)	Significance (p)
STUDY I.				
B.D.	0.532	n.s.	0.603	<0.05
B.M.	0.565	0.05	0.634	<0.05
E.M.	0.735	0.001	0.788	0.001
P.V.	0.277	n.s.	-0.014	n.s.
STUDY II.				
M.B.	0.962	0.001	0.908	0.001
J.C.	0.622	<0.01	0.934	0.001
E.H.	0.886	0.001	0.870	0.001
B.M.	0.860	0.001	0.881	0.001
K.M.	0.877	0.001	0.923	0.001
J.T.	0.889	0.001	0.946	0.001
G.W.	0.956	0.001	0.815	<0.01
Group	0.971	<0.001	0.951	<0.001
STUDY III.				
J.A.	0.202	n.s.		
L.R.	0.708	<0.02	0.812	<0.01
C.v.d.S	0.778	<0.02		
Group	0.694	0.05		

were weaker.

C. FATTY ACID COMPOSITION OF PLASMA AND MILK

1. Fatty Acid Composition of Plasma Plasma fatty acids were analyzed to demonstrate adherence by the subject to a diet which was being followed in an unsupervised, free-living situation. The purpose of the study was to alter plasma fatty acid composition and measure the extent of change in the milk fatty acid composition. The fatty acids in plasma which were measured were the total fasting fatty acids, and were therefore less sensitive to dietary change than those in milk, which were mainly triglyceride fatty acids. Although several subjects did not respond to dietary change as much as had been anticipated, this did not prevent their inclusion in the analysis of results.

The mean values of the major plasma fatty acids are shown in Table 6.7. In any one individual the change was often not statistically significant due to week to week variation. In the whole group, however, increasing the polyunsaturated fatty acid content of the diet, by using polyunsaturated margarine and oils, significantly increased the plasma linoleic acid in Studies I ($p < 0.001$) and II ($p < 0.05$), at the expense of decreased plasma myristic acid, (Study I, $p < 0.05$; Study II, $p = 0.05$; Study III, $p < 0.001$) and palmitic and palmitoleic acids in Study I ($p < 0.05$ and $p < 0.01$ respectively).

The fatty acid composition of plasma in Study I, carried out during the puerperium is shifted in the direction of the shorter chain fatty acids. A similar pattern is seen in the fatty acid composition of milk (Table 6.7).

2. Fatty Acid Composition of Milk In all studies the milk linoleate content increased rapidly in response to a dietary increase. By the fifth day the linoleic acid content had doubled. Table 6.7 shows that the significant increase in linoleate occurred at the expense of myristate (Study I, $p < 0.05$; Study II, $p < 0.001$; Study III, $p < 0.001$) and

TABLE 6.7

Milk and Maternal Plasma Fatty Acid Composition

Study	Diet		Milk Fatty Acids (% of total)										Plasma Fatty Acids (% of total)						
		Saturation of fat**	C8*:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	>20	C12:0	14:0	16:0	16:1	18:0	18:1	18:2
I	A	S		2.8	5.2	8.1	23.7	5.4	9.6	37.3	8.4			1.4	2.7	27.0	6.0	9.6	23.8	30.0
(n=4)	B	P		3.3	3.9	5.4	20.9	5.3	7.9	39.0	15.8			1.0	1.7	24.0	4.8	8.7	22.3	37.8
	C	P		2.8	5.0	6.0	19.5	6.4	7.6	36.6	16.4			2.1	2.2	24.3	5.1	8.7	21.8	35.1
II	D	S	Tr***	Tr	2.6	6.6	25.0	3.7	10.4	42.2	8.7	Tr		1.0	20.5	3.1	9.9	27.2	38.3	
(n=7)	E	P	Tr	Tr	2.7	5.3	21.5	3.5	9.6	42.0	14.7	Tr		0.7	19.2	2.8	10.0	26.3	40.8	
III	F	S		Tr	2.1	5.5	23.9	3.5	9.8	42.9	11.3	0.8	Tr	1.0	19.7	3.1	8.9	26.8	37.9	
(n=3)	G	P		Tr	2.3	4.2	20.9	2.2	9.9	40.5	19.2	0.6	Tr	0.5	19.4	2.8	8.7	23.8	42.3	

*C8:0 = number of carbon atoms followed by number of double bonds

**Saturation of fat: S - rich in saturated fatty acids
P - rich in polyunsaturated fatty acids

***Tr: trace - less than <0.1%

palmitate (Study I, $p < 0.05$; Study II, $p < 0.001$; Study III, $p < 0.01$). Significant changes sometimes occurred in stearic, palmitoleic and oleic acids, but these were not constant.

As mentioned in the previous section, the fatty acid composition of milk during the puerperal study is shifted in the direction of the shorter chain fatty acids.

3. Relationship Between Plasma and Milk Fatty Acids The milk linoleate content generally followed that seen in the fasting plasma (Figure 6.4). A significant overall correlation of 0.721 was obtained for the contents of milk and plasma linoleic acid. Individual variation did occur, particularly when there were only small changes in the P/S ratio of the diet. The individual correlation coefficients are shown in Table 6.8.

D. THE FEEDING INFANT

The plasma cholesterol concentrations of the 7 children who were studied weekly in parallel with their mothers are shown in Table 6.9 and Figure 6.5. Due to the small number of values for any one child, both the means and ranges have been included in the table. A single result from an eighth child (B.M.) is also shown. There was a decrease in the average plasma level in all infants, but this was not always of statistical significance. The decrease in plasma cholesterol was highly significant ($p < 0.001$) for the whole group.

Figure 6.6 shows the serial results from one mother and infant pair (L.R.), with a reciprocal relationship between the milk linoleate content and the plasma cholesterol level of the feeding infant. The response of the infant has a time lag, here seen to be about 1 week. The infant was hypercholesterolaemic at birth (cord plasma concentration 156 mg/100 ml) and at 6 weeks of age had a plasma level of 235 mg/100 ml.

FIGURE 6.4 The Relationship Between the Linoleate Contents of Plasma and Milk

The plasma linoleate content (expressed as a percentage of the total fatty acid) and the resultant milk linoleate in any one subject in each week of the dietary experiments are positively related. The correlation coefficients of individual subjects are shown in Table 6.8.

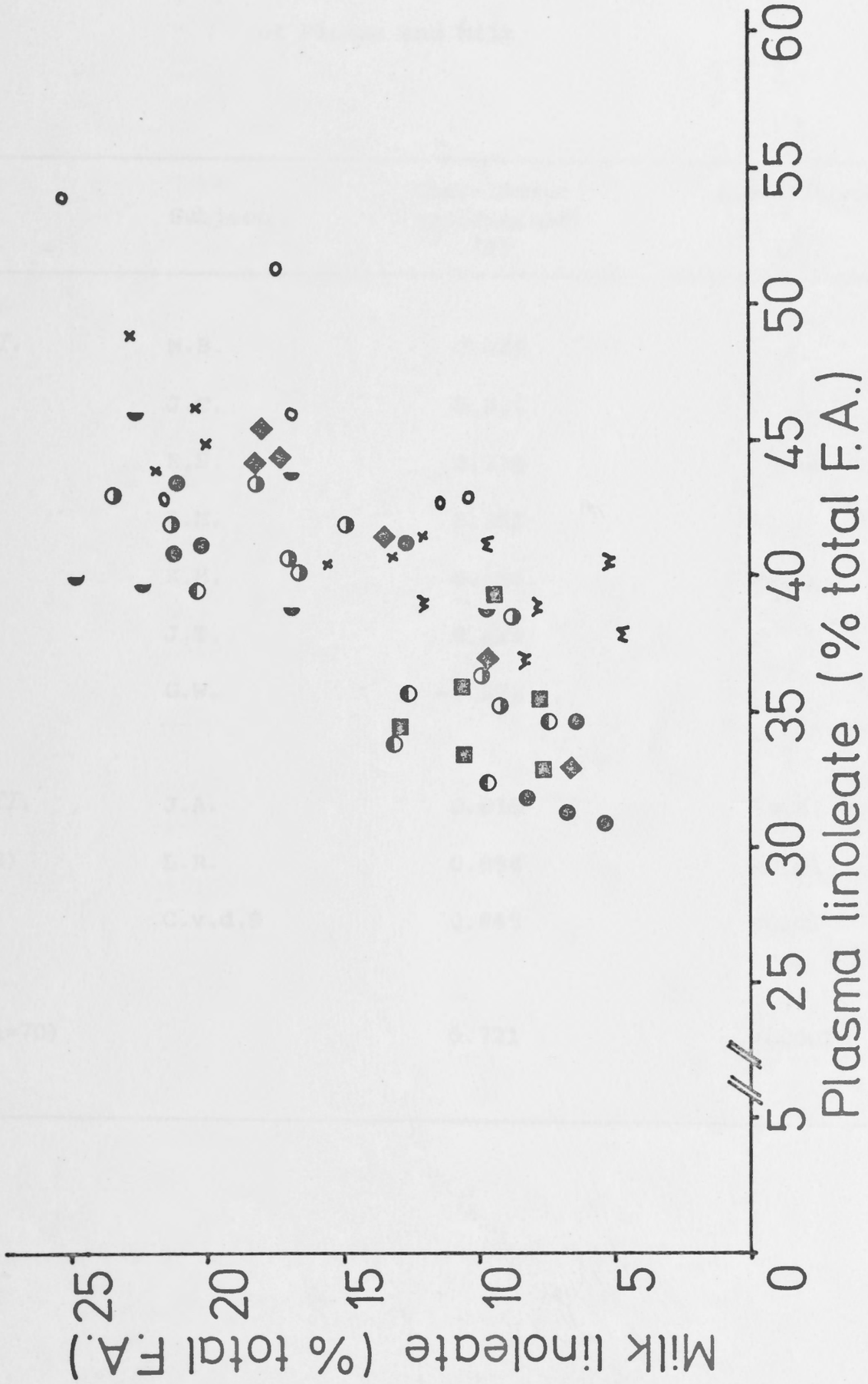

Milk linoleate (% total F.A.)
25
20
15
10
5
0
5
25
30
35
40
45
50
55
60
Plasma linoleate (% total F.A.)

TABLE 6.8

The Relationship Between the Linoleate Contents

of Plasma and Milk

	Subject	Correlation coefficient (r)	Significance (p)
STUDY II.	M.B.	0.019	
(n=6/7)	J.C.	0.331	
	E.H.	0.810	0.05
	B.M.	0.392	
	K.M.	0.984	<0.001
	J.T.	0.619	
	G.W.	-0.273	
STUDY III.	J.A.	0.816	<0.01
(n=10/12)	L.R.	0.854	<0.01
	C.v.d.S	0.845	<0.01
Group (n=70)		0.721	<0.001

TABLE 6.9

The Effect of Changes in the Linoleate Content of Human Milk on the Plasma Cholesterol Level of the Feeding Infant

Subject	More Saturated Diet		More Unsaturated Diet	
	Milk Linoleate*	Plasma Cholesterol**	Milk Linoleate	Plasma Cholesterol
STUDY II.				
(Diet D or E)				
M.B.	8.3	150 (150)	11.3	96 (91-100)
K.M.	8.8	162 (155-169)	17.4	128 (124-132)
J.T.	12.7	163 (156-170)	21.1	158 (144-171)
G.W.	6.4	173 (164-182)	9.3	137 (125-150)
B.M.	5.2	194	7.2	184
STUDY III.				
(Diet F or G)				
J.A.	11.4	195 (194-196)	17.0	189 (161-229)
L.R.	7.3	261 (236-314)	18.4	210 (192-253)
C.v.d.S	15.3	185 (172-192)	22.1	151 (125-167)
Mean ± S.D.		185 ± 35***		156 ± 38***

*Milk linoleate - % of total fatty acid

**Plasma cholesterol - mg/100 ml

***Significantly different at $p < 0.001$

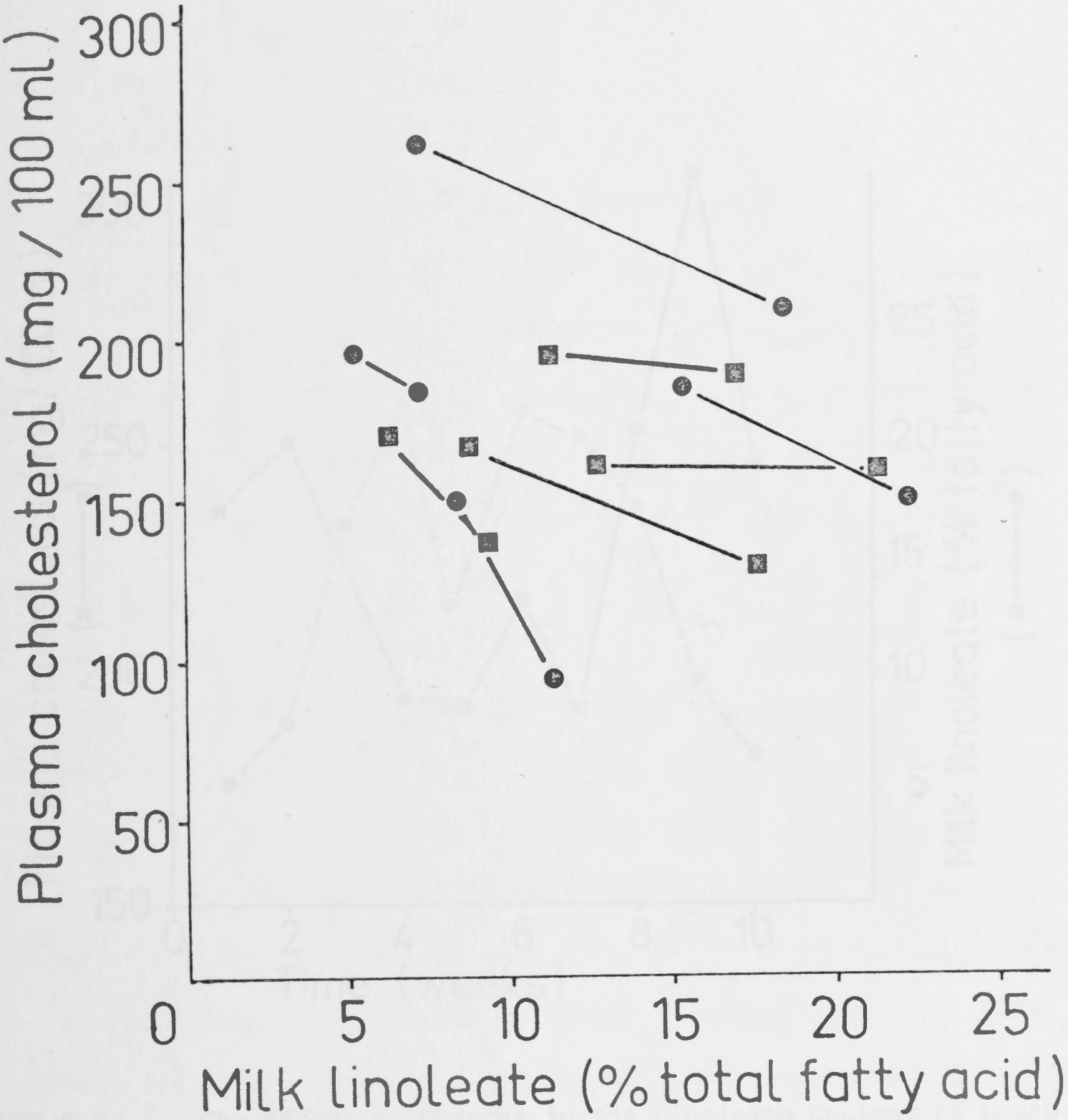


FIGURE 6.5 The Effect of Changes in the Linoleate Content of Human Milk on the Plasma Cholesterol Level of the Feeding Infant

The mean plasma cholesterol concentration of each of 8 infants is shown during the periods in which his or her mother was eating a saturated or more unsaturated diet. The milk linoleate content of each woman during these periods is also shown.

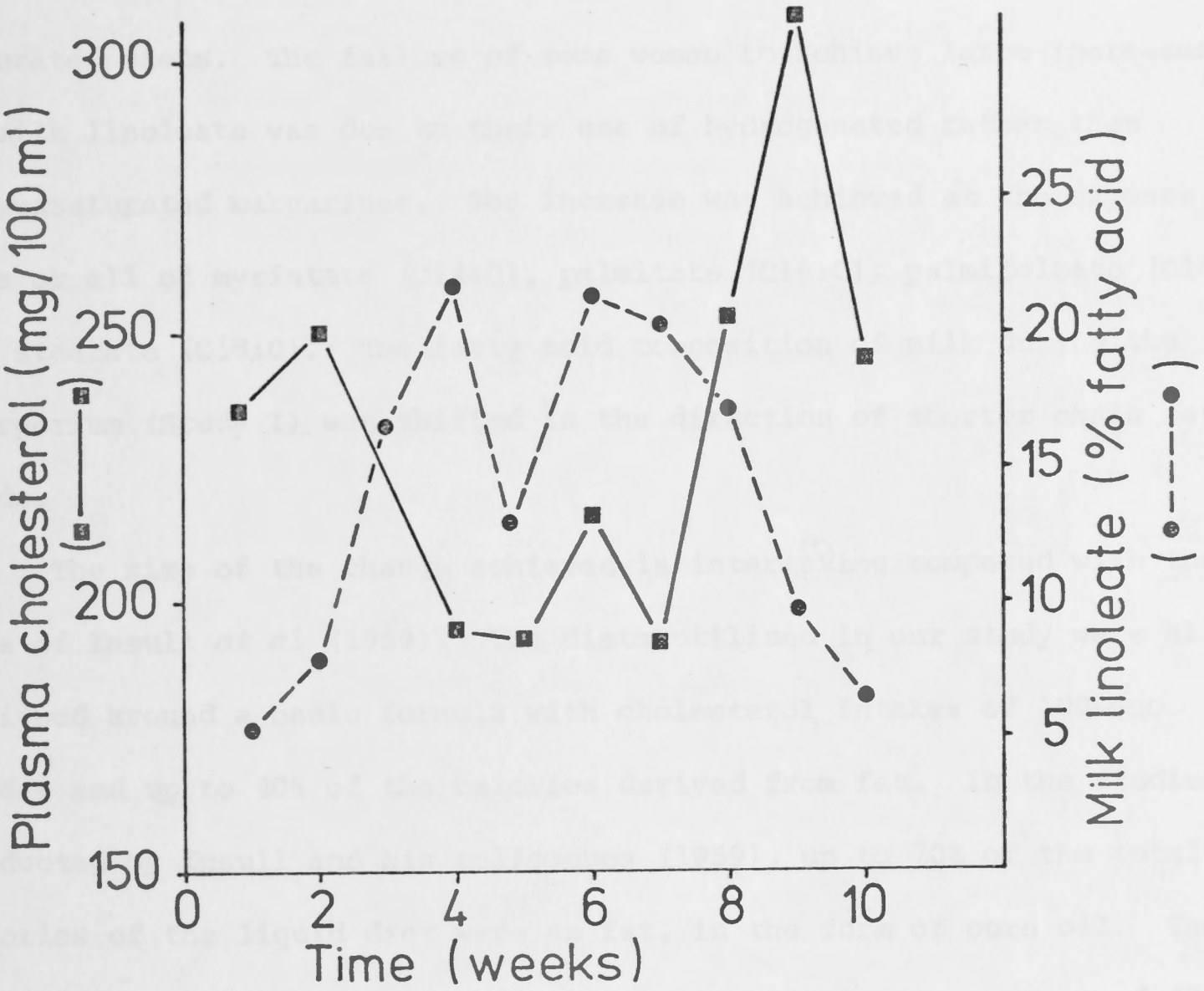


FIGURE 6.6 The Effect of Changes in the Linoleate Content of Human Milk on the Plasma Cholesterol Level of the Feeding Infant: The Longitudional Study of One Infant

The plasma cholesterol concentration of infant (L.R.) was measured weekly from the age of 6 weeks, as was the linoleate content of her mother's milk. During the first 3 weeks, the maternal diet was saturated, for which was substituted an unsaturated diet for the next 6 weeks. For a further 3 weeks she re-substituted the original saturated diet. There was an inverse relationship, which is displaced in time, between the infant's plasma cholesterol concentration and the milk linoleate content.

■——■ Plasma cholesterol concentration (mg/100 ml)

●- - - -● Milk linoleate content (% total fatty acid)

DISCUSSION

A. THE PRODUCTION OF MILK

1. Milk Triglyceride Fatty Acids Increasing the polyunsaturated fatty acid content of the maternal diet was reflected by a rapid increase in milk linoleate. The linoleate and linolenate contents showed a 2-fold increase in all studies when comparing the less saturated with the more saturated diets. The failure of some women to achieve large increases in milk linoleate was due to their use of hydrogenated rather than polyunsaturated margarines. The increase was achieved at the expense of some or all of myristate (C14:0), palmitate (C16:0), palmitoleate (C16:1) and stearate (C18:0). The fatty acid composition of milk during the puerperium (Study I) was shifted in the direction of shorter chain fatty acids.

The size of the change achieved is interesting compared with the data of Insull *et al* (1959). The diets utilized in our study were all designed around a basic formula with cholesterol intakes of 100-600 mg/day and up to 40% of the calories derived from fat. In the studies conducted by Insull and his colleagues (1959), up to 70% of the total calories of the liquid diet were as fat, in the form of corn oil. They were able to attain a maximum linoleate plus linolenate content of 42-44%, expressed as a percentage of the total fatty acids of milk. In a control diet, using the same subject, but providing 40% of the calories as lard, the value for linoleate was 10.3%. This value is similar to that reported for the composition of human milk from free-living subjects on *ad libitum* diets (Insull and Ahrens, 1959). The profiles of milk fatty acid composition vary among different ethnic groups, dietary fatty acid and carbohydrate being important (Read *et al*, 1965a).

The fatty acids of milk are derived from 2 sources; from circulating chylomicrons and lipoproteins of plasma and from endogenous synthesis within alveolar cells of the mammary gland (Linzell and Peaker,

1971). The relative contribution from the two sources has been estimated in animals. Linzell (1968) showed that in the goat, all milk fatty acids up to and including myristate (C14:0), plus some 50% of the palmitate (C16:0) were synthesized from acetate and β-hydroxy butyrate, accounting for some 40% of the total milk fat. The remainder, mainly oleate and stearate (C18) was taken up directly from plasma free fatty acids and triglycerides. In studies with small precursor molecules, both ruminant and rodent mammary tissue have been shown to have the capacity to synthesize short chain fatty acids up to C14, there being some difference in the preferred precursor molecule between species, resulting in characteristic fatty acid patterns (Folley and McNaught, 1961; Garton, 1963; Smith, 1971; Katz and Wals, 1972). In estimating how much of the milk fat was derived from dietary as opposed to endogenous sources, Glascock *et al* (1956) estimated that, in the goat, 27% was obtained directly from the diet. Insull *et al* (1959) studied this by using isocaloric, hypocaloric and hypercaloric diets of varying fatty acid composition. With eucaloric diets, as in the present studies, the fatty acids of the milk reflected those in the diet, but with hypocaloric diets, there was an increase in milk lauric acid, reflecting increased synthesis within the mammary gland. With the hypercaloric diets, there was an increase in milk fat concentration — the dietary fatty acids contributing significantly if the diet contained much fat. If the excess calories were derived from carbohydrate there was an increase in short chain fatty acids due to increased synthesis in the mammary gland.

There may be a functional intermediate lipid pool in the mammary gland which prevents large fluctuations in the fatty acid composition (Insull *et al*, 1959). Such fluctuations do occur in the milk fatty acid composition of women on high carbohydrate diets (Read *et al*, 1965b). In this situation, the relative contributions of short (C12 and C14) and long (C16 and C18) chain fatty acids are determined by the relative

availability of substrate in the plasma: 8 hours after a high carbohydrate meal, the levels of laurate and myristate become maximal. Evidence for the presence of a buffering storage pool is based upon the observation that 2 to 3 days are required before a change in milk fatty acid composition becomes maximal following dietary change (Insull *et al*, 1959). Furthermore, although labelled fatty acid will appear in milk as soon as 4 hours after being given orally, the maximum concentration is not reached for 23 hours (Glascock *et al*, 1956). Against this, fluctuations in fatty acid composition occur during each 24 hour period (Read *et al*, 1965b) and it is known that large amounts of chyle may be directly taken up by the lactating udder of the goat (Lascelles *et al*, 1964).

Study I, carried out during the puerperium, showed fatty acid profiles, in both milk and plasma, which were shifted in the direction of an increased percentage of shorter chain fatty acids, namely C10-C14. This may reflect changes in the composition of fatty acids in plasma during pregnancy (Taylor, 1972), but the variability in the reported compositions for total fatty acids (Degrelle-Cheymol, 1964; Woodruff *et al*, 1964; Renkonen, 1966) or triglyceride fatty acids (Sweeney *et al*, 1963) at term, makes any conclusion difficult. Maternal adipose tissue biopsies at mid-gestation (Roux *et al*, 1971) or at delivery (Sweeney *et al*, 1963) show lower levels of C14:0, C16:1 and C18:1 and higher levels of C16:0, C18:0 and C18:2 compared with normal women (Heffernan, 1964).

2. Milk Cholesterol The cholesterol content of human milk was independent of dietary cholesterol. This is in contradistinction to findings in other species such as the rat (Reiser and Sidelman, 1972) and the guinea-pig (Connor and Lin, 1967), in which the cholesterol content of milk rises with increases in dietary and plasma cholesterol. The source of milk cholesterol is two fold, from plasma and from endogenous

mammary synthesis. The proportion derived from plasma varies between species, being 70-80% in the rat (Chevallier, 1964; Clarenberg and Chaikoff, 1966; Easter, 1971), 35% in the guinea-pig (Connor and Lin, 1967), 50-60% in the goat (Mills *et al*, 1974) and a significant proportion in the rabbit (Connor and Lin, 1967). In the rhesus monkey, approximately two-thirds of milk cholesterol is derived from plasma (Pitkin *et al*, 1972). The contribution from plasma in guinea-pig milk increases 2-fold with increases in dietary cholesterol, which also greatly raised the concentration in plasma (Connor and Lin, 1967). In ruminants fed a protected polyunsaturated oil supplement, there is an increase in the plasma cholesterol concentration (Bitman *et al*, 1973; Mills *et al*, 1974), yet the milk cholesterol content remains unchanged, due to a decrease in the contribution from plasma cholesterol (Mills *et al*, 1974).

In my studies, the failure of the milk cholesterol to rise in the face of an increase in dietary cholesterol may reflect a reduced contribution from plasma as in the ruminant studies, or a compensatory decrease in cholesterogenesis. On the other hand, the decrease in milk cholesterol content with the change in secretion from colostrum to mature milk (Macy and Kelly, 1961) parallels the decrease in post-partum plasma cholesterol levels.

However, the problem may be approached from another point of view. In mature milk, there is a constant relationship between the cholesterol and triglyceride concentrations (Table 6.6), suggesting a functional interrelation. Milk fat consists of lipid globules, primarily triglyceride, surrounded by a hydrophobic cholesterol-phospholipid-containing membrane (Ling *et al*, 1961). The cholesterol content of the bovine membrane comprises some 5.2% of the lipid and the phospholipid 20.4% (Brunner, 1969). The cholesterol in milk may be fractionated into that associated with the fat globules and that remaining in the skim

milk. The latter is associated with membrane particles (Patton, 1973). These consist of microvilli shed from the alveolar cell surface (Stewart *et al*, 1972) and some shed from the fat globule membrane by vesiculation (Wooding, 1971). It has been proposed that the 2 fractions and the cholesterol contained therein may have different origins (Easter, 1973), the cholesterol of the skim milk fraction being derived from the plasma. The turnover of dietary cholesterol from plasma into milk in the rat is 17-20 hours (Easter, 1971), and is found in the skim milk (Easter, 1973). The secretion of milk is dependent upon constant regeneration of cell membranes; protein and lactose secretion utilize membranes in vesicle transport from the endoplasmic reticulum, to the Golgi apparatus and then to the cell surface, where they are secreted by reverse pinocytosis (Helminen and Ericsson, 1968; Keenan *et al*, 1970); triglycerides are secreted in company with hydrophobic substances in globules, being enveloped by portions of the alveolar cell membrane and extruded into the lumen (Hollmann, 1959; Kinsella and McCarthy, 1968). Easter hypothesizes that plasma cholesterol is incorporated into the cell membrane in a regenerative capacity, and is thus indirectly incorporated into milk in its role as a component of the milk fat globule membrane, derived from the alveolar membrane (Patton and Fowkes, 1967). The contribution from this source should therefore be proportional to the secretion of milk, particularly of triglyceride. The relationship found in these studies would support that contention.

The endogenous synthesis of cholesterol within the mammary gland from precursors such as acetate has been demonstrated many times, both *in vivo* (e.g. Popják and Beeckmans, 1950) and *in vitro* (e.g. Clarenberg and Chaikoff, 1966). It is not responsive to negative feedback in the guinea-pig, as demonstrated by Connor and Lin (1967). In the ruminant, cholesterogenesis in the mammary gland is stimulated by polyunsaturated fatty acids (Mills *et al*, 1974). Easter (1973), using mevalonate as

precursor, showed the presence of labelled cholesterol within the milk fat globule fraction only.

Looking at the relationship in Study I more closely, if the correlations are derived for the latter 3 weeks of the study, rather than the entire 4 week period, then the correlation coefficients resemble those of the mature milk samples (Study II). The comparative correlation coefficients are shown in Table 6.10 below.

TABLE 6.10

The Correlation Between Cholesterol and Triglyceride Concentrations in Transitional Milk

Subject	Weeks 1-5 post partum		Weeks 2-5 post partum	
	Correlation coefficient	p	Correlation coefficient	p
B.D.	0.532	—	0.657	<0.05
B.M.	0.565	0.05	0.851	<0.01
E.M.	0.735	0.001	0.947	<0.001
P.V.	0.227	—	0.444	—

3. Milk Phospholipid The question of milk phospholipid has also been considered in terms of 2 pools (Patton, 1973). Most, if not all, phospholipid is synthesized in mammary tissue *de novo* (Easter *et al*, 1971). The pools may relate to the milk fat globule membrane, derived from the plasma membrane which envelopes the globule when secreted, and to the droplet surface itself prior to secretion. The results in Table 6.6 indicate a strong relationship between triglyceride and phospholipid levels. However, since both pools above may well be concentration-dependent and physically determined by the surface area of the globule, such data does not help distinguish between these two possibilities; it

merely denotes a possible functional interaction between the two components.

B. THE CHOLESTEROL LOWERING EFFECT IN THE FEEDING INFANT

The decrease in the infants' plasma cholesterol concentration in response to an increasing concentration of linoleate in milk was variable but within the group the result was significant. There was no relationship however between the total fat or cholesterol content of the milk and the change in infant plasma cholesterol levels. The growth of all infants, in terms of weight, fall within and followed population standards.

Other dietary studies carried out during infancy are not strictly comparable. Studies utilizing changes in milk formulae have exposed all infants on one formula to the same P/S ratio and similar cholesterol intakes (Fomon and Bartels, 1960; Sweeney *et al*, 1961, 1962; György *et al*, 1963; Woodruff *et al*, 1964; Darmady *et al*, 1972). Since the average milk cholesterol content remained constant throughout the present study, the total intake per day will have increased slightly over the study period, though in relation to the baby's weight will have remained fairly constant. Any change in plasma cholesterol may therefore be attributed to an alteration in the P/S ratio of the diet. Only small amounts of cereal were introduced during the study, but it is unlikely that the very small amount of fibre would have affected bile acid secretion (Kritchevsky and Story, 1974).

It is known that the linoleic acid content of plasma (Hansen *et al*, 1964) and on adipose tissue (Sweeney *et al*, 1963) varies with the linoleic acid content of the diet. The increase in adipose linoleate is more rapid in infancy (Sweeney *et al*, 1963) than in the adult (Christakis *et al*, 1965). Plasma cholesterol concentration is lowered by increasing the P/S ratio of the diet (Keys *et al*, 1965; Grande *et al*, 1972; Nestel *et al*, 1973). The study by Nestel *et al* (1973) in adults, using

polyunsaturated ruminant fat is the most comparable, since the diet composition remained unchanged except for the change in fatty acids. The mechanism of action of polyunsaturated fats appears to be through increased cholesterol catabolism and excretion, at least during the initial 3-4 weeks while the plasma cholesterol concentration is falling (Moore *et al*, 1968; Nestel *et al*, 1973), though this may not continue into the new steady state (Grundy and Ahrens, 1970). Infants fed on cholesterol-free soy milk, with a P/S ratio of 4.2 show an increase in bile acid synthesis which is maintained for up to 6 weeks (Chapter 5).

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